Lagovirus europaeus GI.2/RHDV2 (Rabbit Haemorrhagic Disease Virus 2)

Expanding the paradigm of a pathogenic lagovirus

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Cover: The expanded scope of *Lagovirus europaeus* GI.2/RHDV2. Background: Electron micrograph of lagovirus particles (Photo: Dolores Gavier-Widén). Hosts of GI.2/RHDV2 from upper left to lower right: Young and adult wild rabbits (*Oryctolagus cuniculus*) that succumbed to GI.2/RHDV2 (Photo: Aleksija Neimanis). European brown hare (*Lepus europaeus*) (Photo: Karin Bernodt). Mountain hare (*Lepus timidus*) on Hallands Väderö (Photo: Aleksija Neimanis). Domestic rabbit (*Oryctolagus cuniculus*) (Photo: Aleksija Neimanis)

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Abstract

Rabbit haemorrhagic disease (RHD) was first detected in 1984 and quickly spread among wild and domestic European rabbits (*Oryctolagus cuniculus*) throughout the world. RHD and European brown hare syndrome (EBHS), a related disease of hares (*Lepus spp.*), are caused by pathogenic lagoviruses of the Family Caliciviridae. Infection of susceptible animals results in a necrotizing, often fatal, hepatitis. The causative viruses known as *Lagovirus europaeus* GI.1/RHDV and *L. europaeus* GII.1/EBHSV are considered genus specific, causing disease in *Oryctolagus cuniculus* and some *Lepus spp.*, respectively. Full susceptibility to clinical disease only occurs after approximately eight weeks of age.

This understanding of pathogenic lagoviruses changed in 2010 with the emergence of a new virus, *L. europaeus* GI.2/RHDV2. While this new lagovirus shares features with the previously described, classic lagoviruses, it can cross species barriers and caused mortality in two southern European hare species, *Lepus capensis mediterraneus* and *Lepus corsicanus*. It also infects and kills young rabbits.

Studies here explored and helped define this new, expanded paradigm of pathogenic lagoviruses. Susceptibility to GI.2/RHDV2 was described in two new host species, the European brown hare (*Lepus europaeus*) and the mountain hare (*Lepus timidus*). Mortality events in hares in Sweden, Italy and Spain provided insight into disease ecology. Infections likely spilled over from GI.2/RHDV2 outbreaks in rabbits, and the mountain hare outbreak lasted several months in the absence of rabbits. Disease caused by GI.2/RHDV2 was compared to classic RHD and EBHS in young and adult rabbits, and adult hares, respectively. In adults, pathology and virus tissue distribution were the same irrespective of the causative virus. In contrast to GI.1/RHDV, GI.2/RHDV2 caused similar disease in 5-week old kittens and adults. This thesis helped map GI.2/RHDV2 emergence by investigating its presence in Sweden. The first confirmed case was in May 2013, but initial cases were diagnosed as classic RHD. Widespread outbreaks did not occur until 2016 and phylogenetic analysis supported virus evolution and/or multiple introductions. Findings in this thesis helped prevent disease in domestic rabbits in Sweden and further our understanding of GI.2/RHDV2. GI.2/RHDV2 provides an excellent opportunity to study how viruses overcome species and age barriers.

Keywords: rabbit haemorrhagic disease, RHDV2, lagovirus, emerging, pathology, rabbit, *Oryctolagus cuniculus*, *Lepus*, wildlife, virus

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Dedication

To the leporid lovers Johan, Taiga and Meja.

For the rabbits:
My heart has joined the Thousand, for my friend stopped running today.
Richard Adams, Watership Down

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Neimanis, AS*, Ahola, H, Zohari, S, Larsson Pettersson, U, Bröjer, C, Capucci, L, Gavier-Widén, D. (2018). Arrival of rabbit haemorrhagic disease virus 2 to northern Europe: Emergence and outbreaks in wild and domestic rabbits (*Oryctolagus cuniculus*) in Sweden. *Transboundary and Emerging Diseases*, 65, pp. 213-220.

II Velarde, R, Cavadini, P, Neimanis, A, Cabezón, O, Chiari, M, Gaffuri, A, Lavin, S, Grilli, G, Gavier-Widén, D, Lavazza, A*, Capucci, L. (2017) Spillover events of infection of brown hares (*Lepus europaeus*) with rabbit haemorrhagic disease type 2 virus (RHDV2) caused sporadic cases of an European brown hare syndrome-like disease in Italy and Spain. *Transboundary and Emerging Diseases*, 64 (6), pp. 1750-1761.

III Neimanis, A*, Larsson Pettersson, U, Huang, N, Gavier-Widén, D, Strive, T. (2018) Elucidation of the pathology and tissue distribution of *Lagovirus europaeus* GI.2/RHDV2 (rabbit haemorrhagic disease virus 2) in young and adult rabbits (*Oryctolagus cuniculus*). *Veterinary Research*, 49:46, pp. 1-15.

IV Neimanis, AS*, Ahola, H, Larsson Pettersson, U, Lopes, AM, Abrantes, J, Zohari, S, Esteves, P, Gavier-Widén, G. Overcoming species barriers: An outbreak of *Lagovirus europaeus* GI.2/RHDV2 in an isolated population of mountain hares (*Lepus timidus*). (submitted manuscript)

* Corresponding author.

Abbreviations

AEC	3-amino-9-ethylcarbazole
CIBIO	Centro de Investigação em Biodiversidade e Recursos Genéticos
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DAB	3,3'-diaminobenzidine
DIC	disseminated intravascular coagulation
EBHS	European brown hare syndrome
EBHSV	European brown hare syndrome virus
ELISA	enzyme-linked immunosorbent assay
HBGA	histo-blood group antigens
HE	haematoxylin and eosin
Hpi	hours post-inoculation
HRP	horseradish peroxidase
IHC	Immunohistochemistry
IZSLER	Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna 'Bruno Ubertini'
M:E	myeloid to erythroid ratio
MAb	monoclonal antibody
MRCV	Michigan rabbit calicivirus
OD	optical density
OIE	World Organisation for Animal Health
ORF	Open reading frame
PCR	polymerase chain reaction
RCV	rabbit calicivirus
RHD	rabbit haemorrhagic disease
RHDV	rabbit haemorrhagic disease virus
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SCB	Statistiska Centralbyrån

SEFaS	Servai d'Ecopatologia de Fauna Salvatage
SLU	Swedish University of Agricultural Sciences
SVA	National Veterinary Institute

1 Introduction

1.1 Rabbits and hares

The mammalian order Lagomorpha includes rabbits, hares and pikas, which are characterised by their four, continuously growing, incisors in the upper jaw and entirely herbivorous diet. Rabbits and hares are further classified as leporids within the family Leporidae. Three leporids currently inhabit Sweden: the European rabbit (*Oryctolagus cuniculus*), the European brown hare (*Lepus europaeus*) and the mountain hare (*Lepus timidus*). While the rabbit and European brown hare are widely distributed throughout Europe, with the exception of alpine regions further south, the mountain hare is restricted to northern Europe and Asia (Angerbjorn & Flux, 1995).

1.1.1 European rabbit (*Oryctolagus cuniculus*)

The European rabbit is grey-brown with a reddish nape, lives in social groups in warrens and produces altricial young. Gestation is short (30 days) and females can produce multiple, successive litters of 4-5 kittens during the breeding season, making them a fecund species. The European rabbit originates from the Iberian peninsula (Lees & Bell, 2008) and was introduced into southern Sweden at the beginning of the 1900s (Andersson *et al.*, 1979). Although wild populations are thought to be restricted to southern Sweden and the island of Gotland, feral populations have established in cities such as Stockholm. Approximate distribution of wild and feral rabbits in Sweden is depicted in Figure 1. These data represent voluntary observations of wild rabbits collected by ArtDatabanken, Swedish University of Agricultural Sciences (https://www.artdatabanken.se/, accessed June 8, 2018). Population size is unknown but annual hunting bags peaked at more than 450 000 animals in the

early 1960s before decreasing drastically following the introduction of myxomatosis in the 1960s (Svenska Jägareförbundet, 2016). Annual hunting bag was less than 50 000 rabbits in 2015.

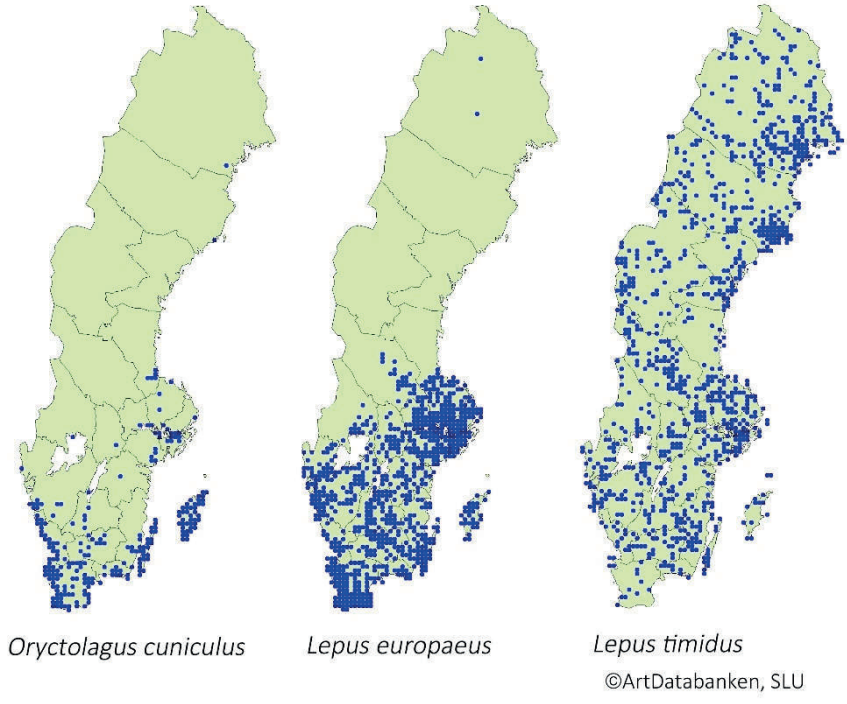

Figure 1. Observations (blue dots) of free-ranging European rabbits (*Oryctolagus cuniculus*), European brown hares (*Lepus europaeus)* and mountain hares (*Lepus timidus*) in Sweden as reported voluntarily by the general public to ArtDatabanken, Swedish University of Agricultural Sciences (https://www.artdatabanken.se/, accessed June 8, 2018). Reproduced with permission.

In addition to free-ranging rabbits in Sweden, domestic rabbits are kept as pets and raised for meat and fur. According to the most recent report from Statistics Sweden (Statistiska Centralbyrån, SCB) in 2012, 2.8% or approximately 77 000 households had at least one pet rabbit (SCB, 2012). Rabbit production in Sweden is small-scale compared to southern Europe, but detailed statistics on rabbit production in Sweden are lacking. The Swedish rabbit producers' association (Sveriges Kaninproducenters förening, SKPF) estimates that 15 000-25 000 rabbits were slaughtered in Sweden in 2016 and rabbit meat currently is being marketed as a climate-friendly alternative to pork and beef (Nilsson, 2017). An unknown number of rabbits are raised in households for personal consumption without being sold.

1.1.2 European brown hare (*Lepus europaeus*)

The European brown hare is the largest of the three leporids in Sweden, weighing between 4-6 kg. It is yellow-brown with yellow irises (Figure 2) and gives birth to precocial young. Gestation period is 45 days and females can give birth to up to three litters per breeding season. The European brown hare was introduced into Sweden at the end of the 19th century and until 1990, the northern distribution was limited by the *limes norrlandicus,* a borderline between the boreal and hemiboreal zones (Jansson & Pehrson, 2007). Its range has since expanded further north (Jansson & Pehrson, 2007) and also overlaps with both rabbits and mountain hares (Figure 1). European brown hares are grazers and prefer agricultural landscapes whereas mountain hares are browsers and prefer forested habitats (Jansson & Pehrson, 2007). Similar to the wild rabbit, population numbers of the European brown hare in Sweden are not known. Hunting bags show a declining trend with annual numbers of almost 125 000 in the 1950s compared to approximately 25 000 in 2015 (Svenska Jägareförbundet, 2016).

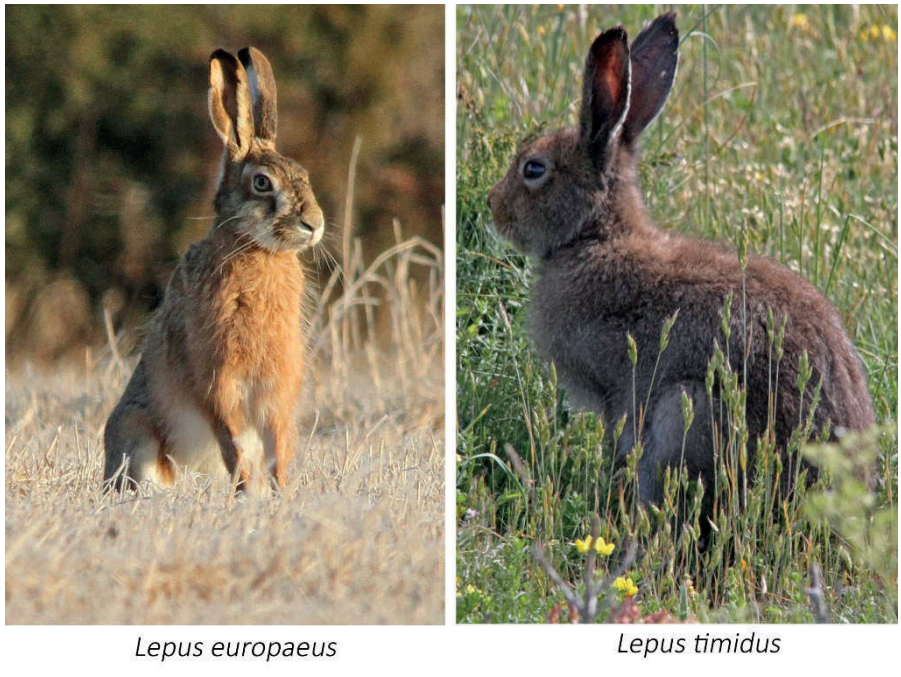

Figure 2. European brown hare (*Lepus europaeus*) and mountain hare (*Lepus timidus*). Photos: Karin Bernodt.

1.1.3 Mountain hare (*Lepus timidus*)

The mountain hare is the only leporid that is considered native to Sweden. It is smaller than the European brown hare, the ears are shorter and the face is rounder, but the hindfeet are bigger (Angerbjorn & Flux, 1995). The summer pelage is grey-brown (Figure 2) and changes to white or grey-white in the winter. Young are precocial. Gestation period is 50 days and females typically give birth to 1-2 litters per breeding season (Angerbjorn & Flux, 1995). Four to five years ago, there were an estimated 600 000 breeding pairs (350 000-850 000) in Sweden (ArtDatabanken, pers comm). Like other leporids, hunting bags have declined. Annual bags peaked at almost 200 000 animals in the 1980s and early 1990s but fell to less than 25 000 in 2015 (Svenska Jägareförbundet, 2016). Mountain hare populations are decreasing in southern Sweden where their range overlaps with European brown hares and populations have completely disappeared from the far south (Thulin, 2003).

1.2 Rabbit Haemorrhagic Disease

1.2.1 History

Rabbit haemorrhagic disease (RHD) is a highly infectious and often fatal viral hepatitis of the European rabbit (*Oryctolagus cuniculus*) (Figure 3). RHD was first described in China in 1984 in Angora rabbits that had been imported from Germany (Xu, 1991) and first detection in Europe occurred in southern Italy in 1986 (Morisse *et al.*, 1991). The causative virus, termed rabbit haemorrhagic disease virus (RHDV), spread rapidly throughout Europe and Asia and made incursions into Africa and the Americas (Morisse *et al.*, 1991). In 1991, the virus was imported into Australia for evaluation as a biocontrol agent to combat highly invasive, introduced European rabbits (Cooke & Fenner, 2002). During field trials on Wardang Island in 1995, the virus escaped to infect mainland rabbits and quickly spread. Since 1996 and 1997, RHD viruses are intentionally used as biocontrol agents against rabbits in Australia and New Zealand, respectively (Cooke & Fenner, 2002). RHD has at some point been documented on every continent except for Antarctica (Abrantes *et al.*, 2012) and is considered endemic in many countries that have wild or feral populations of European rabbits.

Figure 3. A wild rabbit *(Oryctolagus cuniculus*) that died from rabbit haemorrhagic disease. Photo: Aleksija Neimanis.

1.2.2 Epidemiology and transmission

RHD is highly infectious and the virus can be transmitted directly from animal to animal and indirectly via contaminated fomites such as grass, hay, bedding, shoes and clothing. Mortality in susceptible animals is high, around 90%, and death occurs within 48-96 hours post inoculation (hpi) (Marcato *et al.*, 1991). Tissues, secretions and excretions from rabbits with clinical RHD contain huge amounts of virus. For example, liver can have a titre from 10^3 LD50 [50% lethal dose] to $10^{6.5}$ LD50/ml of 10% liver homogenate, (OIE, 2015). The virus remains viable for extended periods, especially within organic material (Henning *et al.*, 2005). It survived for at least 225 days in tissue suspension kept at 4°C (Šmíd *et al.*, 1991) and can withstand freeze-thaw cycles (Morisse *et al.*, 1991). Carcasses are thought to play a key role in environmental persistence as virus can remain viable in carcasses for at least three months (Henning *et al.*, 2005).

The oral route is considered to be the primary mode of transmission (Morisse *et al.*, 1991), but infection can also occur through the nasal, conjunctival, subcutaneous, intramuscular and intravenous routes. Insects can act as mechanical vectors. In a field study in Australia, it was shown that a single flyspot (regurgita or faeces) from flies that had fed on carcasses from an RHD-outbreak can contain enough infectious material (2-3 LD50) to kill a susceptible rabbit (Asgari *et al.*, 1998). Humans, insects, and scavenging birds and mammals can facilitate spread over larger distances. Importation of infected rabbit meat

was believed to be the source of an outbreak in Mexico (House *et al.*, 1990) and the virus most likely escaped Wardang Island, Australia via insects (Cooke & Fenner, 2002).

1.2.3 Clinical signs

Infected animals develop pyrexia. Disease can be peracute and animals die without showing clinical signs. In acute disease, depression, anorexia, nasal discharge, neurological abnormalities and respiratory signs may be seen. Icterus may be observed in subacute disease (Marcato *et al.*, 1991).

1.2.4 Pathology and pathogenesis

RHD manifests itself as a necrotizing hepatitis typically accompanied by disseminated intravascular coagulation (DIC). Grossly, the liver is light brown or tan and friable and has an accentuated lobular pattern (Figure 4). The spleen may be dark red and enlarged and lungs often are oedematous with variable congestion and haemorrhage (Figure 4). Petechial haemorrhages may be observed in other tissues and rarely, icterus is seen. Microscopically, there is periportal to massive hepatic necrosis. Splenic necrosis, lymphoid depletion and multisystemic fibrin thrombi, particularly within the kidneys and lungs, are variably seen (Fuchs & Weissenbock, 1992; Marcato *et al.*, 1991).

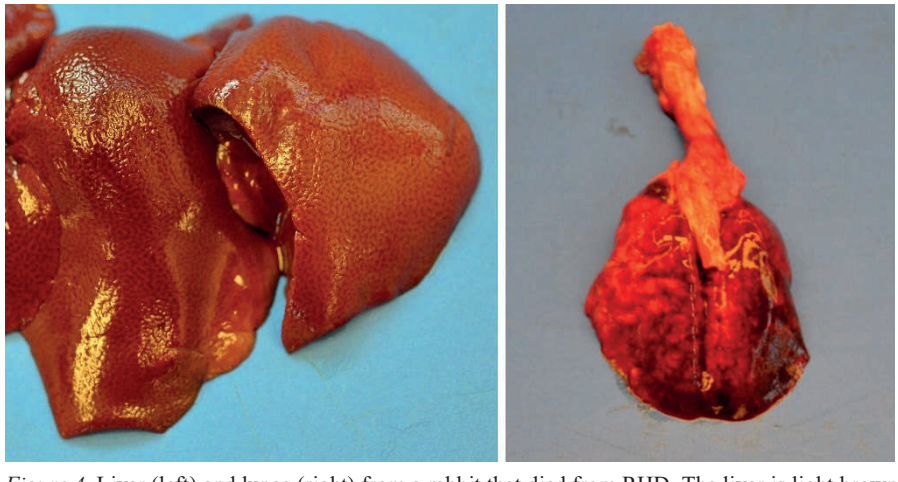

Figure 4. Liver (left) and lungs (right) from a rabbit that died from RHD. The liver is light brown and has a reticular pattern and the lungs are oedematous, congested and haemorrhagic. Photos: Aleksija Neimanis.

Initiation of infection is not completely understood. The virus has been shown to bind to histo-blood group antigens (HBGA) which are expressed on rabbit epithelial cells of the small intestine and upper respiratory tract (Ruvoen-Clouet *et al.*, 2000). These cells likely represent the most common site of viral entry, but infection can also occur subcutaneously, intramuscularly and intravenously.

Once the virus has entered the host, it quickly targets the liver. Viral antigen was detected within hepatocytes as early as 8 and 12 hpi (Prieto *et al.*, 2000; Gelmetti *et al.*, 1998). Infection begins periportally at the periphery of lobules and then expands throughout the lobule (Jung *et al.*, 2000; Park *et al.*, 1995). Heterophils often accompany degenerate and dead hepatocytes (Fuchs & Weissenbock, 1992). Apoptosis is thought to be a key mechanism in hepatocellular death (Jung *et al.*, 2000; Alonso *et al.*, 1998) and areas of lytic necrosis can also be seen (Park *et al.*, 1995).

Following viral antigen detection in hepatocytes, antigen may be observed in Kupffer cells and in macrophages in other tissues, particularly the spleen. There are inconsistent reports of antigen detection in other cells such as endothelial cells (Alonso *et al.*, 1998), mesangial cells (Prieto *et al.*, 2000; Stoerckleberger *et al.*, 1992) and bile duct epithelium (Carrasco *et al.*, 1991).

The pathogenesis of DIC in RHD has not been definitively elucidated. Proposed mechanisms include sequelae to severe liver disease (impaired synthesis of coagulation proteins and protease inhibitors, and reduced clearance of activated factors) (Ueda *et al.*, 1992), activation of extrinsic factors by necrotic hepatocytes, or direct endothelial damage (Marcato *et al.*, 1991).

1.2.5 Diagnosis

Microscopic lesions of RHD are pathognomonic (Fuchs & Weissenbock, 1992). If tissues are fresh enough for examination, a diagnosis of RHD can be made using histology only, but confirmation of virus presence requires additional testing. There are a variety of methods available to demonstrate presence of virus in tissues. Virus particles can be observed using negative staining electron microscopy of liver homogenate (Capucci *et al.*, 1991) and viral antigen can be demonstrated *in situ* in tissues by immunohistochemistry (IHC) (Stoerckle-Berger *et al.*, 1992) or *in situ* hybridization (Gelmetti *et al.*, 1998). Although evaluation of haemagglutination of human erythrocytes has been used to detect virus, some strains do not haemagglutinate (OIE, 2016). Viral antigen can be detected in samples using various enzyme-linked immunosorbent assays (ELISAs) (Capucci *et al.*, 1991) and numerous polymerase chain reactions (PCR) have been developed to detect viral RNA in samples (Bascunana *et al.*,

1997; Guittre *et al.*, 1995). Different serological assays are used to detect previous exposure or vaccination (Capucci *et al.*, 1991). Because the causative virus cannot be grown in cell culture, rabbit inoculations are still required to propagate virus and demonstrate that the viral material present is infectious.

1.2.6 Impact and importance

RHD has had severe impacts on wild rabbit populations and broader effects have also been documented in ecosystems where rabbits are a keystone species (Delibes-Mateos *et al.*, 2009). In the Mediterranean basin, rabbits provide essential ecosystem services. In addition to managing flora by grazing, creating habitats for other species by digging warrens, and fertilizing soil through their latrines, they also serve as prey for more than 30 species of predators (Delibes-Mateos *et al.*, 2008). The re-emergence of RHD on the Iberian peninsula led to a 60-70% decrease in rabbit populations that was followed by decreases in fecundities of the endangered Iberian lynx (*Lynx pardinus*) and Imperial eagle (*Aguila adalberti*) by 65.7% and 45.5%, respectively (Monterroso *et al.*, 2016).

RHD has also been a heavy burden on domestic rabbit production prior to the development and implementation of effective vaccines (Arguello Villares, 1991). Over 140 million domestic rabbits succumbed in the 1984 outbreak in China (reviewed in McIntosh *et al.*, 2007) and according to McIntosh *et al.* (2007), by 2007, RHD had killed almost a quarter billion free-living and domestic rabbits. European rabbits are also of significant ecological and economic concern in Australia, but for different reasons. These introduced, highly invasive herbivores are regarded as major ecological and agricultural pests. In stark contrast to Europe and elsewhere, RHD viruses are deliberately released into Australian and New Zealand ecosystems as biocontrol agents to decrease and suppress rabbit populations (Cooke & Fenner, 2002). In a review of the economic value of rabbit control in Australia, in which RHD plays a major role, approximately $70 billion Australian dollars in benefits was estimated for the agricultural industry (Cooke *et al.*, 2013) from 1950-2011. Ecological impacts of rabbit control have also been dramatic (Cooke & Fenner, 2002) but are more difficult to quantify. Because of the highly infectious nature of RHDV and the devastating impact of RHD, RHD was officially added to the OIE list of notifiable diseases in 1989 (Morisse *et al.*, 1991). Measures to prevent RHD in domestic rabbits include effective biosecurity and vaccination.

1.3 Lagoviruses

1.3.1 Pathogenic lagoviruses

The causative agent of RHD remained elusive for the first few years after detection, hampered by the inability to grow the virus in cell culture. It was initially proposed to be a picornavirus (Lee & Park, 1987) or parvovirus (Gregg & House, 1989), but was eventually defined as a calicivirus (Ohlinger & Thiel, 1991). The International Committee on Taxonomy of Viruses (ICTV) recognises Rabbit Haemorrhagic Disease Virus (RHDV) as a species within the genus *Lagovirus,* Family Caliciviridae. Recently, a new nomenclature for lagoviruses has been proposed (Le Pendu *et al.*, 2017) and is being adopted. All lagoviruses are grouped within a single species, *Lagovirus europaeus*. RHDV-like viruses are assigned to genogroup I (GI) and RHDV is further assigned to genotype 1 i.e. *L. europaeus* GI.1/RHDV. The GI.1 genotype is further divided into variants, of which, for example, GI.1a represents the antigenic variant also known as RHDVa (Capucci *et al.*, 1998). A schematic of the proposed classification of pathogenic lagoviruses is presented in Figure 5. A table of current and proposed new names for lagoviruses used in this thesis can be found in Appendix 1. The shortened name of GI.1/RHDV is used throughout the remainder of the thesis.

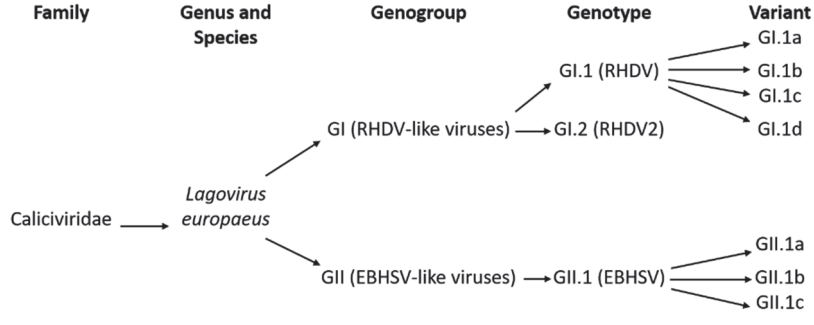

Figure 5. Proposed classification of pathogenic lagoviruses according to Le Pendu *et al.* (2017).

GI.1/RHDV is a small (35-40 nm) non-enveloped, single-stranded, positive sense RNA virus with an outer capsid protein layer surrounding the RNA core. The genomic RNA molecule is 7437 nucleotides long (Gould *et al.*, 1997). The genome is organized into two overlapping open reading frames (ORFs), ORF1 encodes a polyprotein which is post-translationally processed into nonstructural proteins (NSPs) that are essential for virus replication and the major structural protein, the capsid VP60 (reviewed in Abrantes *et al.*, 2012). ORF2 encodes

VP10, a minor structural protein. The GI.1/RHDV genome also encodes 5′ and 3′ untranslated regions (5′UTR and 3′UTR, respectively). The VP60 capsid gene sequence is approximately 1 740 nucleotides in length and is useful for the inference of phylogenetic relationships of lagoviruses (Kinnear & Linde, 2010). Characteristic of caliciviruses (Figure 6), GI.1/RHDV has 32 cup-shaped depressions on the capsid surface and icosahedral symmetry.

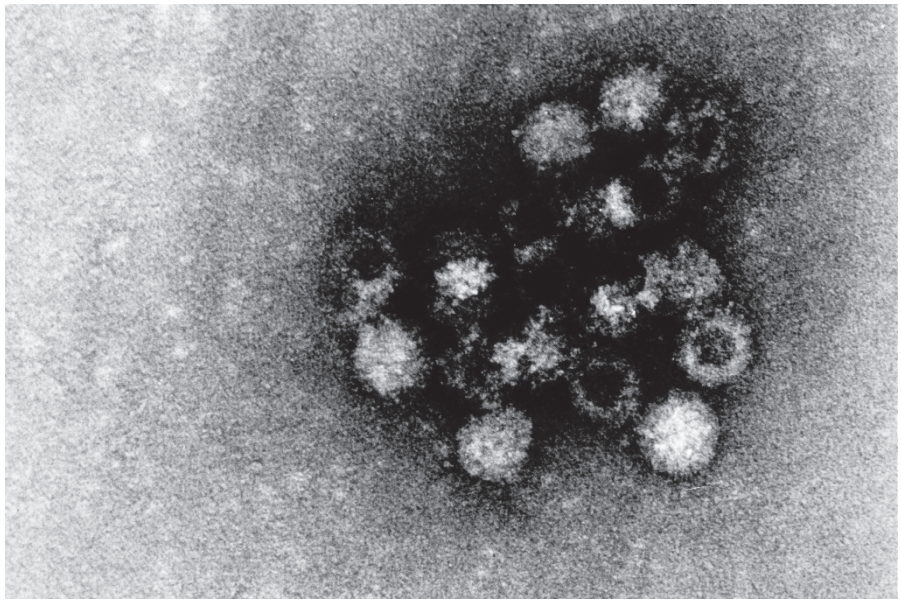

Figure 6. Lagovirus particles as observed by negative staining electron microscopy of liver homogenate. Photo: Dolores Gavier-Widén.

In addition to GI.1/RHDV, another pathogenic lagovirus emerged in European brown hares in the 1980s. This related virus causes European brown hare syndrome (EBHS) and has been assigned to genogroup II, genotype 1, i.e. *Lagovirus europaeus* GII.1/EBHSV (Figure 5, Appendix 1). Although GII.1/EBHSV can infect and cause disease in other species, including the mountain hare, it does not infect European rabbits.

GI.1/RHDV and GII.1/EBHSV share many similarities that have defined the paradigm of a pathogenic lagovirus. Both viruses target the liver, causing pathognomonic lesions of periportal to midzonal, or even massive, apoptosis and necrosis. Morbidity and mortality of susceptible animals is high and disease tends to be primarily peracute to acute, with a smaller proportion of subacute to chronic infections. GI.1/RHDV and GII.1/EBHSV are generally considered to be genus-specific, with disease confined to the European rabbit and various hare species, respectively. In addition to their restricted host range, age range of

susceptible animals is also sharply delimited. Young animals are naturally resistant to development of clinical disease. Susceptibility begins after five weeks of age and full susceptibility to infection and disease only occurs after eight weeks of age. This restricted age range has been best studied in the rabbit and possible, although not necessarily mutually exclusive, hypotheses to explain this phenomenon include differences in HBGA viral receptor expression in young and adult rabbits (Ruvoen-Clouet *et al.*, 2000), innate immunity (Marques *et al.*, 2014) and changes in liver function at 5-6 weeks of age (Morisse *et al.*, 1991).

1.3.2 Non-pathogenic and variably pathogenic lagoviruses

In addition to pathogenic lagoviruses, non-pathogenic viruses have also been described in European rabbits. These include rabbit calicivirus, RCV (Capucci *et al.*, 1996), 06-11/RCV-E1 (Le Gall-Recule *et al.*, 2011) and RCV-A1 (Strive *et al.*, 2009), the latter of which have been newly classified as *L. europaeus* GI.3 and GI.4, respectively, according to Le Pendu et al. (2017) (Appendix 1). These viruses do not cause clinical disease and notably, they have a different tissue tropism than their pathogenic cousins. The intestine, rather than the liver, appears to be the target organ (Hoehn *et al.*, 2013; Capucci *et al.*, 1996). Immunity against some, but not all, of these viruses provides variable cross-protection against GI.1/RHDV infection. Still other lagoviruses (e.g. Michigan rabbit calicivirus, MRCV) have been described to have variable pathogenicity (Bergin *et al.*, 2009).

1.3.3 A new pathogenic lagovirus

Lagovirus europaeus GI.2/RHDV2 (rabbit haemorrhagic disease virus 2)

In 2010, a new lagovirus was detected that challenged the current understanding of pathogenic lagoviruses. This new virus, recently classified as *Lagovirus europaeus* GI.2/RHDV2 (Le Pendu *et al.*, 2017) (Figure 5) (Appendix 1) is also called RHDV2 (Le Gall-Recule *et al.*, 2013) or RHDVb (Dalton *et al.*, 2012). It shares an average nuclear identity of 82.4% with GI.1/RHDV and GI.1a/RHDVa viruses (Le Gall-Recule *et al.*, 2013). It is antigenically distinct from GI.1/RHDV and can therefore partially evade protective immunity against GI.1/RHDV viruses. This is significant because vaccines developed against GI.1/RHDV are at best only partially protective against GI.2/RHDV2 infection. First detection occurred in August 2010 when an unexpected outbreak of RHD occurred in vaccinated animals in a rabbitry in France (Le Gall-Recule *et al.*,

2013). Through retrospective analysis, the first documented cases of GI.2/RHDV2 occurred in France in domestic and wild rabbits in April and May 2010, respectively (Le Gall-Recule *et al.*, 2013).

By 2011, GI.2/RHDV2 was already detected on the other side of the Pyrenees and Alps in both Spain (Dalton *et al.*, 2012) and Italy (Le Gall-Recule *et al.*, 2013) and significant epidemiological differences between early GI.2/RHDV2 strains and classic GI.1/RHDV and GII.1/EBHSV strains were observed. In Spain, there were descriptions of clinical disease and death in animals as young as 11 days of age (Dalton *et al.*, 2012), challenging the paradigm that full susceptibility was only reached after eight weeks of age. In Italy, another departure from the classic paradigm of a pathogenic lagovirus was observed when GI.2/RHDV2 infected and killed not only rabbits, but also Sardinian Cape hares (*Lepus capensis mediterraneus*) (Puggioni *et al.*, 2013) and an Italian hare (*Lepus corsicanus*) (Camarda *et al.*, 2014). On-going wildlife surveillance programs in both Spain and Italy also identified three European brown hares with RHD- or EBHS-like disease after detection of GI.2/RHDV2 in these countries. Furthermore, experimental work with these early GI.2/RHDV2 strains supported a lower and more variable pathogenicity and longer disease course when compared with GI.1/RHDV (Le Gall-Recule *et al.*, 2013). Some early strains (e.g. 10-32 in France) caused little to no mortality in experimental infections (Le Gall-Recule *et al.*, 2013). Despite these significant differences from GI.1/RHDV and GII.1/EBHSV, descriptions of pathology and tissue distribution of GI.2/RHDV2 that may provide additional insight into lagovirus pathogenesis have been few and brief (Duarte *et al.*, 2015; Le Gall-Recule *et al.*, 2013; Dalton *et al.*, 2012).

GI.2/RHDV2 continued to spread, reaching mainland Portugal in 2012 (Abrantes *et al.*, 2013), northern Europe in 2013, and to date, has been reported throughout the majority of western Europe, including remote island archipelagos such as the Azores (Duarte *et al.*, 2015) and the Canary Islands (Martin-Alonso *et al.*, 2016). Notably in 2015, GI.2/RHDV2 jumped continents and was detected in wild rabbits in Australia (Hall *et al.*, 2015), where it continues to spread and is now the dominant field strain (Mahar *et al.*, 2017). Also of significance was the outbreak of RHD in Finland in 2016 caused by GI.2/RHDV2 (Isomursu *et al.*, 2018), which was the first documentation of RHD ever in Finland. The outbreak occurred in a relatively recently established, feral population of rabbits in the greater Helsinki area and at least one domestic rabbit also succumbed. The establishment of feral rabbit populations can allow for introduction and maintenance of GI.2/RHDV2 in regions where it otherwise would be unable to proliferate as was also recently observed on Vancouver Island in Canada in 2018 (OIE WAHID, 2018).

1.4 RHD in Sweden

RHD was first detected in wild rabbits on the island of Gotland in February 1990 (Gavier-Widén, 1993) before reaching the mainland in 1993 (Wiss, 1993). Dramatic epizootics in the first years following incursion were eventually replaced by few, sporadic cases occasionally reported in wild rabbits. This low level of RHD continued until March 2016 when the situation significantly changed with the onset of frequent and widespread outbreaks.

At the beginning of this thesis, in addition to gross and histologic examination, routine diagnostic tests available at the National Veterinary Institute (SVA) to demonstrate the etiological agent of RHD included nested PCR and IHC. However, none of the above methods distinguish between different viruses that can cause RHD and additional diagnostic methods were required and developed. In addition to collaborating with the Department of Microbiology, SVA to set up a GI.2/RHDV2-specific RT-qPCR for research purposes, an improved IHC method was developed and optimised to aid in diagnosis and to better study tissue distribution and pathogenesis of GI.2/RHDV2.

This thesis investigated the arrival and emergence of GI.2/RHDV2 in Sweden because of the consequences this could have on wild and domestic leporid populations. It also furthers our understanding of this new, emerging virus and the disease it causes.

2 Aims of the thesis

This thesis focused on both the emerging virus *Lagovirus europaeus* GI.2/RHDV2 and the disease that it causes. These were represented by two main aims:

- Investigate the presence of and gain a better understanding of the genetic diversity of GI.2/RHDV2 strains in Sweden
- Help define the expanded scope of GI.2/RHDV2 through investigations of disease in previously known and newly documented susceptible host species and age groups

Specific objectives were:

- Investigate the arrival and emergence of GI.2/RHDV2 in Sweden (**I**)
- Investigate the phylogenetic relationships of GI.2/RHDV2 strains found in rabbits, European brown hares and mountain hares in this thesis to gain further insight into origins and epidemiology (**I**, **II**, **IV**)
- Document the expanded host range of GI.2/RHDV2 through first description of GI.2/RHDV2 in European brown hares and mountain hares (**II**, **IV**)
- Describe the gross and microscopic pathology and virus tissue distribution of GI.2/RHDV2 in rabbits and hares (**II**, **III**, **IV**)
- Compare the pathology and tissue distribution of GI.2/RHDV2 with classic GI.1/RHDV in young rabbits (**III**)

- Compare the pathology of GI.2/RHDV2 and GII.1/EBHSV in European brown hares and mountain hares (**II**, **IV**)

- Study disease outbreaks to provide insight into the disease ecology of GI.2/RHDV2 (**II**, **IV**)

3 Materials and Methods

3.1 Study animals

Animals and tissues used in this thesis came from diagnostic submissions to veterinary laboratories (studies **I**, **II**, **IV**), regulated hunts (studies **II** and **IV**), breeding for restocking grounds (study **II**) and from a captive-bred research colony (study **III**).

Table 1. *Sample sizes of animals and tissues used for each study*

	Rabbits	European brown hares	Mountain hares
Study **I**	N = 15 fallen rabbits	-	-
Study **II**	-	N = 3 fallen hares N = 662 sera	-
Study **III**	N = 7 inoculated rabbits N = 1 control	-	-
Study **IV**	N = 10 fallen rabbits N = 11 genetic sequences from study **I**	-	N = 3 fallen hares N = 6 hunted hares

3.1.1 European rabbits (*Oryctolagus cuniculus*)

*Diagnostic submissions (Sweden) (**I, IV**)*

Animals in study **I** included all wild and domestic rabbits with RHD submitted to SVA's wildlife disease surveillance program and SVA's diagnostic pathology service for domestic species from May 2013 to May 2016. These included case material from 11 wild rabbits (n = 7 incidents) and from four domestic rabbits (n = 3 incidents).

In study **IV**, 10 rabbits were selected from the RHD cases submitted to SVA between June 2016 and June 2017 to complement the phylogenetic analysis of lagoviruses in mountain hares. Data from 11 rabbits from study **I** were also included.

*Domestic rabbits (Australia) (**III**)*

Eight captive-bred, domestic rabbits used in study **III** originated from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) research colony in Canberra, Australia. Animals consisted of three adults and five 5-week old kittens.

3.1.2 European brown hares (*Lepus europaeus*)

The European brown hares examined by necropsy in study **II** (n = 3) were found dead and submitted to diagnostic laboratories in Italy (n = 1) and Spain (n = 2). For the serosurvey in study **II**, sampling of animals differed between Spain and Italy. In Spain, serum was collected from European brown hares that were taken in the regulated hare hunt (n = 106), whereas in Italy, serum was collected from live animals caught in areas managed as 'breeding for restocking grounds' (n = 407). Additionally, archived sera collected in Italy from 2007-2010 (n = 149 samples) were included as control sera prior to the detection of GI.2/RHDV2 in Italy in 2011.

3.1.3 Mountain hares (*Lepus timidus*)

The mountain hares in study **IV** came from a small, isolated population on the island of Hallands Väderö (N 56° 26.6', E 12° 33.7) off the southwest coast of Sweden. The three hares examined by necropsy were part of a mortality event and submitted to SVA's wildlife disease surveillance program. Tissues were also collected from hares hunted on Hallands Väderö during (n = 4) and after (n = 2) the mortality event.

3.2 Sample collection and experimental study design

3.2.1 Natural GI.2/RHDV2 infection

Sweden

Swedish wildlife that are found dead or euthanised can be voluntarily submitted to the National Veterinary Institute (SVA) for examination free of charge. Carcasses are examined by necropsy as part of a long-standing, national wildlife disease surveillance program. This passive surveillance is based on opportunistic submission of samples. Data, fresh tissues and any paraffin-embedded, formalin-fixed tissues are archived for future use. SVA also has a diagnostic pathology service for domestic animals. Throughout this thesis project, press releases in traditional, electronic and social media were used to inform hunters, rabbit owners and the general public about emerging GI.2/RHDV2 and to solicit reports and submissions of dead rabbits.

Study **I** was designed to make use of archived and prospective case material from SVA's wildlife disease surveillance program and prospective case material from SVA's diagnostic pathology service to investigate the presence and emergence of GI.2/RHDV2 in Sweden. GI.2/RHDV2 was first detected in France in 2010, therefore a database search of all archived RHD incidents in wild rabbits from 2009 to the beginning of the study in 2015 was performed. Eight incidents (n = 13 rabbits) were identified and of those, three incidents from 2009-2012 were confirmed by genotyping to have been caused by GI.1/RHDV. These three incidents were therefore excluded from the study. The remaining five incidents and all prospective cases of RHD in wild and domestic animals submitted to SVA until May 2016 were included, giving a total of 15 rabbits from ten separate incidents.

All rabbits, with the exception of one domestic rabbit for which only liver was submitted, underwent post-mortem examination and a suite of tissues including liver, spleen, lung, kidney, brain, colon and muscle were frozen at -20°C for molecular analyses and fixed in 10% neutrally buffered formalin for microscopic examination. RHD was diagnosed based on gross and microscopic lesions of acute necrotizing hepatitis accompanied by detection of lagovirus material in the liver by nested PCR (Bascunana *et al.*, 1997) or IHC (see section 3.3.1). These diagnostic analyses for RHD do not distinguish between GI.1/RHDV and GI.2/RHDV2. Therefore, liver or spleen from at least one rabbit

per incident was analysed using a GI.2/RHDV2-specific RT-qPCR (section 3.3.2). If lagovirus material was found, the virus was genotyped following amplification and sequencing of the VP60 gene. Immunological typing of the lagovirus was also performed on livers of three rabbits (section 3.3.3).

In study **IV**, reports of a mortality event in mountain hares on Hallands Väderö began in September 2016 when a wide-spread outbreak of GI.2/RHDV2 was occurring in rabbits on the adjacent mainland (Fig 8, section 4.1.2). With help from the local hunting association, three hares found dead in November 2016, December 2016 and March 2017 were collected, frozen and submitted to SVA. Animals were examined by necropsy and a suite of tissues including liver, spleen, lung, kidney, bone marrow, brain, small intestine, colon, heart, trachea, salivary gland, nasal mucosa and skin were collected in 10% neutrally buffered formalin for histological examination and virus localization using IHC. Not all tissues were collected from all animals and autolysis ranged from moderate to severe. Liver from all three animals was frozen at -20°C and -70°C for genotyping and immunotyping of any lagoviruses present. It was also used for molecular identification of hare species (n = 2) and bacterial culture (n = 1).

To investigate presence of virus in apparently healthy mountain hares on Hallands Väderö during and after the outbreak, tissues were collected during the regulated hunt in January 2017 (n = 4) and September 2017 (n = 2), respectively. Liver and duodenum were collected in the January hunt and stored at -20°C and liver, spleen, duodenum, jejunum, ileum, sacculus rotundus and caecal appendix were collected in 10% neutrally buffered formalin for histology and IHC. Fresh and formalin-fixed liver samples were collected in the September 2017 hunt.

Mountain hares are the only leporid species on Hallands Väderö and phylogenetic analysis of the lagovirus strains in mountain hares was conducted to gain insight into their origin. From June 2016 to June 2017, rabbits from 62 confirmed RHD incidents were examined by SVA's wildlife and domestic pathology services. Of these, samples from 10 incidents representing broad geographical and temporal range were selected for inclusion in the phylogenetic analysis. Using PCR, the VP60 gene was amplified from liver samples and sequenced (section 3.3.2). VP60 sequences from study **I** were also included, as were other publicly available *L. europaeus* GI sequences.

Italy and Spain

In study **II**, three European brown hares were found dead with EBHS-like disease in Italy and Spain after the detection of GI.2/RHDV2 in rabbits in both countries. Animals were submitted to regional diagnostic veterinary laboratories for investigation (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna 'Bruno Ubertini' (IZSLER), the location of the OIE

Reference Laboratory for RHD, in Brescia, Italy and Servai d'Ecopatologia de Fauna Salvatage (SEFaS) in Barcelona, Spain). Liver from all three hares was collected for immunological typing and genotyping. Liver kidney, lung, spleen and gastrointestinal tract were also collected for bacteriological and parasitological analyses in Italy. In Spain, liver, kidney, lung, spleen, brain, heart and trachea were fixed in 10% neutrally-buffered formalin for microscopic examination.

To gain insight into the presence and occurrence of pathogenic lagoviruses in European brown hares from the regions where GI.2/RHDV2 infection was found, serosurveys were carried out on sera from hares collected during the same time period and in the same region as the cases. Banked sera stored at IZSLER in Brescia, Italy from European brown hares collected before GI.2/RHDV2 was detected in Italy were included as a reference to help interpretation of results.

3.2.2 Experimental GI.2/RHDV2 infection

Because of the expanded age range of GI.2/RHD2, apparent variation in pathogenicity of GI.2/RHDV2 strains and lack of detailed microscopic and immunohistochemical investigations for this viral infection, a detailed histopathological and immunohistochemical study was performed. Naturally infected animals have varying degrees of post-mortem autolysis and concurrent pathology. Therefore, material from experimental inoculations of a small number of purpose-bred rabbits was utilized. The inoculations were done within a research program on RHD in Australia. Procedures were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2013) and were approved by the CSIRO Ecosystem Sciences Animal Ethics Committee (permits ESAEC 13-10, DOMRAB).

GI.2/RHDV2 distinguishes itself from GI.1/RHDV viruses in its ability to cause clinical disease in young rabbits. Two adults and five 5-week old kittens were therefore inoculated with the first GI.2/RHDV2 strain detected in Australia (also known as BlMt-1). Inoculations were performed *per os* using 0.5 ml (kittens) or 1 ml (adults) of clarified 2% w/v liver homogenate of BlMt-1 containing 3 X 10^9viral copies/ml. Rabbits were monitored twice daily or more frequently as required and euthanized when they reached humane endpoints for terminal RHD. A healthy, uninfected adult rabbit from the same colony was sacrificed to serve as a negative control. Duplicate sets of a full suite of tissues (liver, gall bladder with bile, spleen, lung, heart, kidney, thymus, mesenteric lymph node, duodenum, jejunum, ileum, Peyer's patches, appendix, sacculus rotundus, caecum, colon, trachea, femoral bone marrow and brain) were collected from adults and a subset of tissues (liver, spleen, lung, heart, kidney,

duodenum, jejunum, ileum, Peyer's patches, appendix and colon) were collected from the two kittens with known times of death. One set of tissues was frozen at -20°C for molecular analyses and the other was fixed in 10% neutrally buffered formalin for microscopic analyses. For the other three kittens that were found dead, only one subset of tissues was collected and it was frozen at -20°C for molecular analyses. Serum was collected from all animals. Viral load was calculated for all tissues and all tissues were examined microscopically for lesions. Additionally, for bone marrow sections, myeloid:erythroid ratios (M:E) were determined and proportions of early versus later stage myeloid cells were estimated. Viral antigen distribution within tissues was investigated using IHC.

3.3 Laboratory diagnostics and data analyses

3.3.1 Histology and immunohistochemistry

Histological examination was carried out in all studies (**I, II**, **III**, **IV**) and IHC was performed in studies **I**, **III**, **IV**. Following fixation in formalin, tissues were processed for routine microscopic examination. Deparaffinised sections 3-4 µm thick were stained with Mayer's haematoxylin and eosin (Bancroft & Cook, 1984). In study **IV**, von Kossa histochemical stain (Lillie, 1965) was applied to liver sections to visualize calcium deposits.

IHC to detect viral antigen in tissues was performed the same way for studies **I** and **IV** and methods were slightly modified in study **III**. IHC was carried out on liver from all animals in studies **I** and **IV**, on spleen, lung, bone marrow and kidney from fallen hares in study **IV**, and on small intestine from hares hunted in January 2017 in study **IV**. In study **III**, IHC was performed on all available formalin-fixed, paraffin embedded tissues. IHC was done using an automated AutostainerPlus (Agilent Technologies Sweden AB, Kista, Sweden). Three to four µm sections of paraffin-embedded, formalin-fixed tissues were deparaffinised and rehydrated. Endogenous peroxidase activity was blocked by applying hydrogen peroxidase (Dako REAL™ Peroxidase-Blocking Solution, Agilent Technologies Sweden AB, Kista, Sweden) for five minutes. In studies **I** and **IV**, but not study **III**, antigen was then demasked by treatment with proteinase K (Dako Proteinase K, Agilent Technologies Sweden AB, Kista, Sweden) for six minutes. For all studies, slides were then treated with 2% bovine serum albumin (BSA) for 20 minutes to decrease non-specific staining. Slides were then incubated with a 1:700 dilution of a primary monoclonal antibody cocktail (3H6+6G2 IgG1 mouse monoclonal antibodies, 0.32 mg/ml concentration), kindly provided by the OIE Reference Laboratory for RHD,

Brescia, Italy, that targets the capsid protein of lagoviruses (Capucci *et al.*, 1995). Bound antibodies were visualized with the polymer detection system Dako EnVision+ System-HRP Labelled Polymer (anti-mouse) (Agilent Technologies Sweden AB, Kista, Sweden) for 30 minutes followed by application of either the chromogen 3,3'-diaminobenzidine (DAB) (DAKO Liquid DAB+ Substrate Chromogen System, Agilent Technologies AB, Kista, Sweden) or 3-amino-9-ethylcarbazole (AEC) (Dako AEC+ High Sensitivity Substrate Chromogen, Agilent Technologies Sweden AB, Kista, Sweden) for 10 minutes. Mayer's haematoxylin was applied for five minutes as a counterstain. A duplicate section was incubated with a non-specific antibody (Negative Control Mouse IgG1, Agilent Technologies Sweden AB, Kista, Sweden) diluted to the same protein concentration as the primary antibody to serve as the negative immunoglobulin control. A positive control (liver from a rabbit or European brown hare positive by PCR for GI.2/RHDV2 or GII.1/EBHSV, respectively) and a negative control (rabbit or European brown hare liver negative by PCR for lagoviruses) was included on each slide.

3.3.2 PCR, sequencing and phylogenetic analysis

PCR was used in all studies (**I**, **II**, **III**, **IV**) and sequencing of the lagovirus VP60 gene was done in studies **I**, **II** and **IV**. Methods and primers varied according to the research application and laboratory where the work was performed.

RNA extraction

For extraction of RNA in Sweden (studies **I** and **IV**), RNA was extracted from liver or spleen using a Magnatrix 8000+ robot (NorDiag AB, Hägersten, Sweden) and the NorDiag Vet Viral NA (NorDiag AB) extraction kit. In study **II**, although a different method of RNA extraction generally following Boom *et al.* (1990) and Gomez-Laguna *et al.* (2010) for formalin-fixed, paraffin-embedded tissues was performed at SVA, PCR was repeated on higher quality material (fresh liver) at the OIE reference laboratory for RHD in Brescia, Italy. In Italy (study **II**), RNA was extracted using Trizol Reagent (Quiagen, Milano, Italy). In study **III**, extractions were performed at CSIRO in Canberra, Australia with RNeasy mini kit (Qiagen, Chadstone Centre, VIC), the Maxwell 16 LEV simplyRNA tissue kit (Promega, Alexandria, NSW) or the PureLink viral RNA/DNA mini kit (Life Technologies, Scoresby, VIC) depending on the tissue analysed. Molecular species identification of hares in study **IV** was performed at CIBIO in Portugal and DNA was extracted with the EasySpin Genomic DNA Minipreps Tissue Kit (Citomed, Portugal). All extraction kits were used according to the manufacturer's instructions.

PCR to detect lagovirus material in tissues

To facilitate execution of study **I**, a GI.2/RHDV2-specific RT-qPCR was set up at SVA and this same PCR was used in study **IV**. The PCR amplified a 127-bp region of the VP60 gene using methods and primers in Duarte *et al.* (2015b) with the following modifications: the AgPath One Step QPCR mix (Applied Biosystems, Foster City, CA) was used according to manufacturer instructions and the reaction was performed with 2 µl RNA in a total volume of 15 µl.

In study **II**, GI.2/RHDV2 and GII.1/EBHSV-specific RT-PCR were performed in Italy using the One Step RT-PCR Kit (Qiagen, Milano, Italy). Primers used for EBHSV detection were EBHS-148F (5'-GTTGCAGCATCTGTTGCCACTGCGG-3') and EBHS-578R (5'-TGTACACACTCACGACGAGYGTTGGG-3'). Primers for GI.2/RHDV2 RNA detection were: Fra109-F (5'-ACTACTAGCGTGGTCACCACC-3') and Fra567-R (5'-TTGTTATAAACGCTCAGGACCAAC-3').

For study **III**, a universal lagovirus RT-qPCR that detects all lagoviruses known to be present in Australia was used to detect and quantify *L. europaeus* GI.2/RHDV2 tissue loads. Reactions were performed with the SensiFAST SYBR No-ROX One-Step mix (Bioline, Alexandria, NSW, Australia) as described in Hall *et al.* (2017).

Sequencing

For studies **I**, **II** and **IV**, the VP60 capsid gene of GI.2/RHDV2 was sequenced for genotyping and phylogenetic analysis. In studies **I** and **IV**, sequencing was performed at SVA. Four overlapping amplicons were generated using four sets of primers to cover the entire VP60 gene (Table 2). The first primer was taken from Le Gall-Recule *et al*. (2013) and the others were designed manually following alignment of the publicly available GI.2/RHDV2 sequences in the GenBank (Genetic Sequence Database at the National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/genbank).

Table 2. *Primer sets used to sequence the VP60 capsid gene of Lagovirus europaeus GI.2/RHDV2 at SVA.*

Primers for *L. europaeus* GI.2/RHDV2	
F1 5'- GTCGTCTCGGTAGTACCTG	R1 5'-GTTGCCTGTCGGGTGGTAC
F2 5'- ACAATTCCCTCATGTTGTCATTG	R2 5'- GGGTTGTCAGCTGCAGACC
F3 5'- GCGGTTTGCCGCCATTGAC	R3 5'- GTTAGCTGAACCGGCCTCAG
F4 5'- CAGCCAAACAGGATTGTCAATG	R4 5'- CCTGCAAGTCCCAATCCAAC

The sequencing in study **II** was performed in Italy. The VP60 gene was sequenced using the three primer sets detailed for GI.2/RHDV2 UD11 in Le Gall-Recule *et al*. (2013). For all three studies, Sanger sequencing was then carried out on the purified, amplified PCR products.

In study **IV**, molecular species identification of hares was performed by sequencing the nuclear genes UCP2 and IGHG and the mitochondrial gene cytb using primers described in Melo-Ferreira *et al.* (2009) and Pinheiro *et al.* (2014).

*Genotyping and phylogenetic analysis (**I, II, IV**)*

To identify homologous sequences and examine degree of identity with other publicly available sequences in GenBank, a Basic Local Alignment Search (BLASTN, http://www.ncbi.nlm.nih.gov/BLAST/) was performed on generated VP60 sequences.

According to Kinnear & Linde (2010), the full VP60 capsid gene sequences of GI/RHDV-related viruses provide enough information for robust phylogenetic analyses. For all three studies, evolutionary relationships between generated VP60 gene sequences and other publicly available lagovirus VP60 gene sequences were investigated. Sequences were aligned using Clustal W algorithm (Thompson *et al.*, 1994) before generating phylogenetic trees using MEGA 6: Molecular Evolutionary Genetics Analysis (Tamura *et al.*, 2013). Methods and nucleotide substitution models varied for each study. In study I, the Tamura-Nei model and Maximum Likelihood method were used. Study II employed the Kimura 2-parameter model and neighbour-joining method. The GTR+G+I model and Maximum Likelihood method was used in study IV. Statistical support for nodes was estimated using 1000, 1000 and 2000 bootstraps for studies **I**, **II** and **IV**, respectively.

In study IV, phylogenetic analysis was also performed for molecular species identification of mountain hares using MEGA 6. Pairwise-deletion and the p-distance method were used with 500 replicates.

3.3.3 ELISA (immunological typing and serosurvey)

*Immunological typing (**I, II, IV**)*

All immunological typing of lagoviruses was performed by the OIE Reference Laboratory for RHD in Brescia, Italy. Subtyping sandwich ELISAs as described in OIE (2016) were performed on liver samples from all animals submitted (n = 3 rabbits, n = 3 European brown hares, n = 3 mountain hares). Briefly, rabbit hyperimmune sera specific for GI.1/RHDV and GI.2/RHDV2 and hare

hyperimmune serum specific for GII.1/EBHSV were adsorbed to ELISA plates. Samples (1/5 and 1/30 dilution) were then added and incubated to bind any lagovirus present. Four sets of monoclonal antibodies (MAbs) specific for GI.1/RHDV, GI.1a/RHDVa, GI.2/RHDV2 and GII.1/EBHSV were then applied. Rabbit IgG anti-mouse IgG (H+L) labelled with horseradish peroxidase (HRP) was then added to detect bound MAbs, followed by o-phenylenediamine as substrate. Optical density (OD) measurements at least 0.15 greater than the OD of the negative control were considered positive.

*Serosurvey (**II**)*

All sera were tested for lagovirus antibodies at the OIE Reference Laboratory for RHD using the competitive ELISA (cELISA) procedure described in OIE (2016). Two cELISAs were used: one specific for GII.1/EBHSV and one for GI.2/RHDV2. Just as for the sandwich ELISA, hyperimmune serum specific for the virus in question was adsorbed onto the plate. Four dilutions of sera (1/10, 1/40, 1/160, 1/640) were then incubated with the specific virus on the plate. A specific MAb for the virus in question conjugated with HRP was then added to measure how much of the added virus bound to the plate. This was done by measuring the OD. Serum with specific antibodies against the virus would bind up virus, resulting in less virus available to bind to the plate, resulting in a lower OD. The OD of samples at the 1/10 dilution had to be less than 75% of the OD of the negative control serum to be considered positive. The titre of the positive sample corresponded to the dilution at which a 40-60% reduction of OD compared to the negative control was seen. A ratio of the GII.1/EBHSV titre to the GI.2/RHDV2 titre was calculated to help facilitate interpretation of serological results in light of decreased specificity when using serum samples from the field and when GII.1/EBHSV titres are low.

4 Results and Discussion

While GI.2/RHDV2 shares many characteristics with GI.1/RHDV and GII.1/EBHSV, its broader age and host ranges significantly expand the classic paradigm of a pathogenic lagovirus. The results of this thesis help define this broader paradigm by investigating two new susceptible host species of GI.2/RHDV2, examining the molecular epidemiology of the causative viruses and epidemiological contexts in which disease occurred, and documenting the pathology and viral tissue distribution of GI.2/RHDV2 in both previously known and newly documented susceptible host species and age groups. Results of this thesis also help map the emergence of GI.2/RHDV2 by investigating its arrival to Sweden.

4.1 Arrival and emergence of GI.2/RHDV2 in Sweden

4.1.1 May 2013 to April 2016 (Study I)

From May 2013 to the end of 2015, six incidents of RHD were diagnosed in wild and domestic rabbits (Figure 8, section 4.1.2). At the end of March 2016, an incident of RHD occurred in domestic rabbits in Enköping municipality, approximately 75 km northwest of Stockholm and in April 2016, three more incidents of wild and domestic rabbits dying from RHD were reported in central Stockholm and the adjacent municipality of Solna (Figure 8, section 4.1.2.). These cases marked the beginning of a significant and widespread outbreak of RHD in wild and domestic rabbits in the southern half of Sweden in 2016 (see section 4.1.2.).

Using GI.2/RHDV2-specific RT-qPCR, viral material from GI.2/RHDV2 could be detected in animals from all incidents and lagovirus identity was further confirmed by genotyping and immunological typing. Thus, the earliest

documented case of GI.2/RHDV2 in Sweden occurred on 22 May 2013 on the island of Gotland. However, these early cases were not recognized as GI.2/RHDV2 because case history and lesions mimic GI.1/RHDV infections and diagnostic methods to distinguish the two viruses were not available in Sweden at the time. Additionally, outbreaks were sporadic and presented like cases of endemic GI.1/RHDV in Sweden.

From May 2013 to March 2016, GI.2/RHDV2 outbreaks were represented by few, isolated incidents, never associated with more than three animals. They were also widely dispersed geographically, from Gothenburg in the west to Stockholm in the east and occurred on the Baltic islands of Gotland and Öland (Figure 8, section 4.1.2.). Despite these repeated, sporadic events, GI.2/RHDV2 did not cause significant epizootics until spring 2016. Reasons for this sudden increase in GI.2/RHDV2-linked mortalities are not known and virus, host and environmental factors likely all played a role. Phylogenetic analysis provides some possible insight into virus-dependent factors.

VP60 sequence data from GI.2/RHDV2 strains in Sweden generated in study **I** clustered into three separate groups based on time and geographic location (see sequences labelled Paper **I** in Figure 7). Strains from the islands of Gotland and Öland cluster together, while strains from outbreaks on the mainland before 2016 and during 2016 clustered into two additional groups, respectively (Paper **I** sequences in Figure 7). Notably, the sequence from an outbreak in Solna municipality in 2014 did not cluster together with the sequences from the outbreaks in Solna in 2016. Rather, all four sequences from March and April 2016 clustered together in a highly supported group, suggesting that the strains in 2016 were different from previous strains. This suggests viral evolution over time, multiple introductions into Sweden, or likely both. When considering the additional VP60 sequence data (labelled Paper **IV** in Figure 7) that were generated for study **IV**, the more recent GI.2/RHDV2 strains from 2016 and 2017 form at least one more highly supported, distinct group. This further supports the hypothesis that GI.2/RHDV2 has been introduced into Sweden multiple times.

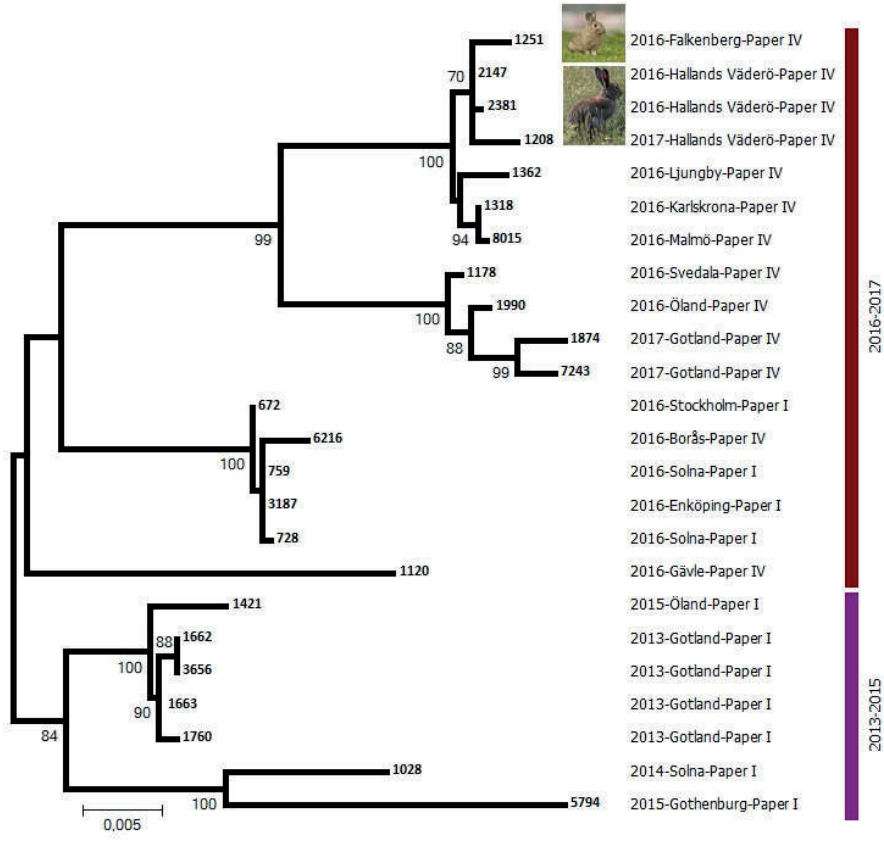

Figure 7. Phylogenetic analysis of VP60 gene sequences of GI.2/RHDV2 strains from rabbits and mountain hares in Sweden (studies I and IV). The phylogenetic tree was generated using Mega 6 software (Tamura et al., 2013), using the maximum likelihood method and the Tamura-Nei model with a gamma distribution. 2000 bootstrap replicates were used to estimate statistical support for nodes. Only the bootstrap values ≥ 70 are shown in the tree. With the exception of sequences 2381, 2147 and 1208 that came from mountain hares, all sequences came from European rabbits.

The presence of GI.2/RHDV2 in Sweden had significant implications for free-ranging rabbits that were naïve to this new virus and domestic rabbits for which effective vaccines were not available (see outbreaks described in section 4.1.2). The potential impact on hare species in Sweden was not yet known.

4.1.2 May 2016 and onwards

After the initial detection and description of GI.1/RHDV2 in Sweden (study **I**), confirmed RHD events were tracked using data from SVA's wildlife disease surveillance program and the diagnostic pathology service for domestic animals.

A separate mortality event occurred on Gotland in May and by June, RHD was detected in Gävleborg county to the north and Västra Götaland and Halland counties to the west (Figure 8). Epizootics swept south and then east during the summer and early fall. By December 2016, outbreaks were confirmed in Stockholm county again (Figure 8). In total during 2016, 61 outbreaks of RHD involving 100 rabbits were confirmed by histology, IHC and/or PCR. This represents only those cases that were submitted to SVA for diagnosis and, based on anecdotal reports, they represent a gross underestimate of the true number of animals that succumbed.

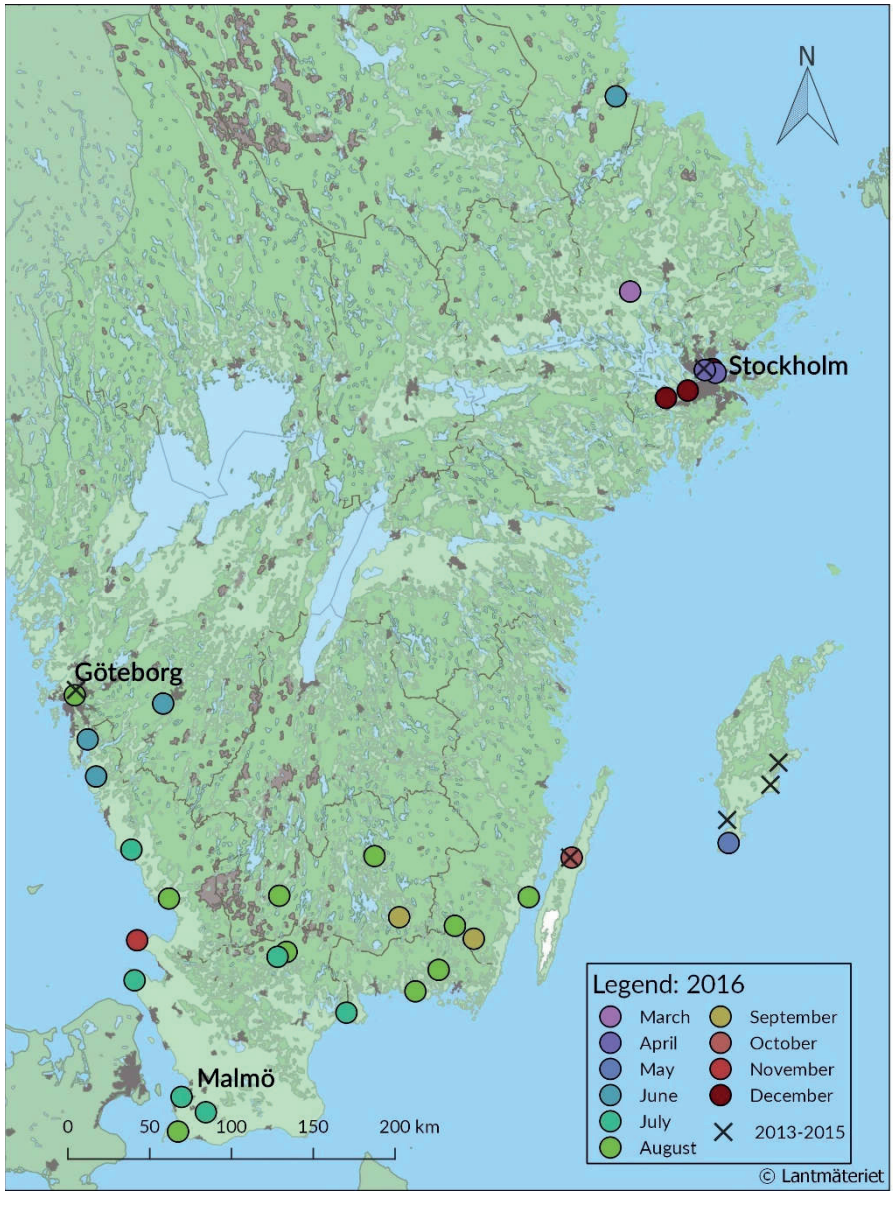

Figure 8. A map of southern Sweden depicting the first confirmed case of rabbit haemorrhagic disease caused by GI.2/RHDV2 per municipality during 2016. Earlier cases of GI.2/RHDV2 from 2013-2015 are also depicted (X).

SVA received fewer reports and rabbits for examination in 2017 and very few to date in 2018. RHD was confirmed in 18 incidents and 23 animals in 2017 and in one incident involving two animals up until June 1, 2018. This decrease in cases most likely represents a true decrease, as animals that survived infection

in 2016 would have developed immunity against re-infection. Additionally, traditional and social media helped inform domestic rabbit owners of this new virus and preventative measures against exposure and infection were widely shared. In the fall of 2016, a GI.2/RHDV2-specific vaccine became more readily available in Sweden, which also helped protect domestic rabbits. However, because SVA relies on voluntary reporting and submission of cases, some proportion of the decrease in cases likely stemmed from waning interest from the general public following the identification of the agent responsible for the mortality events, as well as the availability of an effective vaccine.

Since the first documented case of GI.2/RHDV2 in Sweden in May 2013, all RHD events where the virus has been typed have been caused by GI.2/RHDV2. This suggests that like in France (Le Gall-Recule *et al.*, 2013), the Iberian peninsula (Dalton *et al.*, 2014) and Australia (Mahar *et al.*, 2017), GI.2/RHDV2 is replacing GI.1/RHDV. GI.2/RHDV2 presumably has had a competitive advantage because any pre-existing immunity against GI.1/RHDV in wild and domestic rabbits is only partially protective at best. Another probable competitive advantage stems from the ability of GI.2/RHDV2 to cause clinical disease in younger animals when compared to pre-existing GI.1/RHDV strains. Cooke & Fenner (2002) review how presence of young, naturally resistant rabbits can drive the epidemiology of RHD infection in a given population because they can survive exposure and develop immunity, allowing them to eventually join the breeding population and contribute to future recruitment. Continued monitoring of RHD outbreaks and typing of the responsible lagovirus are necessary for implementation of appropriate vaccination strategies and identification of new variants or viruses.

4.2 New susceptible host species for GI.2/RHDV2

While GI.1/RHDV and GII.1/EBHSV are believed to have rigid host barriers, the results presented in this thesis show that in addition to the European rabbit, the European brown hare and mountain hare can also be infected by GI.2/RHDV2 and develop clinical disease indistinguishable from EBHS. These studies were the first to document GI.2/RHDV2 in these hare species.

4.2.1 European brown hares (Study **II**)

Following detection of GI.2/RHDV2 in both Spain and Italy, three dead European brown hares with EBHS-like disease were examined by necropsy. All three had typical gross lesions of EBHS-like disease including a friable, tan liver, pulmonary haemorrhage (Figure 9) and a variably enlarged spleen grossly. For

the two animals examined microscopically, both had hepatocellular necrosis, acidophilic bodies and fatty degeneration in the liver which are typical microscopic lesions of EBHS. Surprisingly, GI.2/RHDV2, rather than GII.1/EBHSV, was detected in all three animals.

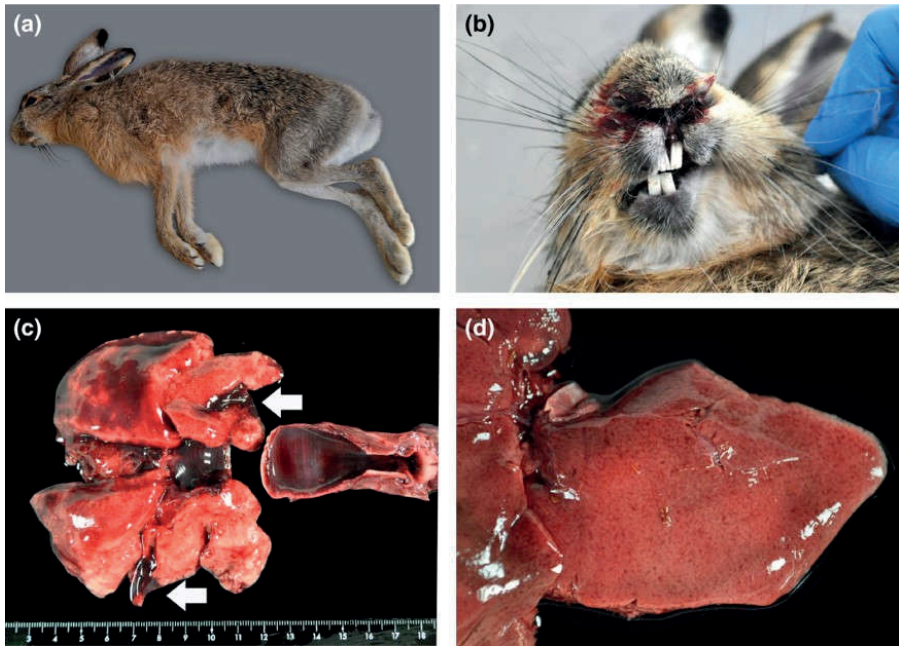

Figure 9. Specimen, epistaxis and macroscopic lesions in a brown hare found dead in Barcelona Province, Spain. (a) One of the European brown hares found dead in Barcelona Province, Spain; (b) epistaxis; (c) diffuse hyperaemia of the tracheal mucosa and multifocal haemorrhages in lungs (arrows); and (d) generalized reticular pattern in the liver suggestive of zonal vacuolar hepatocellular degeneration and necrosis (pictures taken after sampling the tissue). Figure reproduced from Velarde *et al.* (2017), Transboundary and Emerging Diseases under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

No direct source of GI.2/RHDV2 could be identified when the lagovirus VP60 gene sequences from infected hares were compared with available sequences from rabbits. However, hare strains in Spain and Italy were also different from each other. This speaks against the emergence of a hare-adapted GI.2/RHDV2 strain circulating amongst European brown hares, but given the limited dataset, further monitoring and data are required to definitively refute this possibility. In a more recent publication of GI.2/RHDV2 in European brown hares in France (Le Gall-Reculé *et al.*, 2017), cases in hares appeared to be much more common, but hare strains always grouped phylogenetically with local rabbit strains rather than in their own unique cluster. This further supports infection of European brown hares through spillover of infection from rabbits.

Concurrent epizootics of GI.2/RHDV2 were documented in rabbits in Spain and Italy when the three European brown hares died from GI.2/RHDV2. Ranges of rabbits and European brown hares overlap, especially in Spain, and environmental load of GI.2/RHDV2 was likely high. Despite this, only three cases of GI.2/RHDV2 had been reported in Spain and Italy at the time of the study. Because not all dead wild hares will be found and submitted, some cases of GI.2/RHDV2 presumably went undetected. Nevertheless, the serological results from Spain and Italy and absence of reports of larger-scale mortality events in European brown hares support only occasional infections of GI.2/RHDV2 in European brown hares in these two countries. Results varied depending on the time period and country of sampling, but approximately 20-45% of hunted and stocked hares showed very low titres (usually ≤ 1/20) of antibodies against GI.2/RHDV2. While a small proportion of these cases may represent true infections with GI.2/RHDV2, the low titre values can also arise from cross-reactions with antibodies against other lagoviruses, decreased sensitivity of the analysis in field samples, chronic infections and re-infections, and technical margin of error associated with reproducibility of the cELISA. To conclude, results from this study suggest European brown hares most likely are sporadic, spillover hosts of GI.2/RHDV2 within these specific ecological contexts in Italy and Spain. The disease mimics EBHS and additional genetic or immunological techniques must be used to identify the causative lagovirus.

4.2.2 Mountain hares (Study **IV**)

Three carcasses were examined from a mortality event in mountain hares on the island of Hallands Väderö. Similar to the European brown hares of study **II**, all three had hepatic, pulmonary and splenic lesions consistent with EBHS-like disease (Figure 10). The liver was variably friable, discoloured and had an accentuated lobular pattern, there was moderate to severe pulmonary congestion and oedema with variable haemorrhage, and the spleen was enlarged.

Figure 10. A mountain hare *(Lepus timidus*) from Hallands Väderö that died from GI.2/RHDV2 infection. Left: The mountain hare displays characteristic features of the species including relatively short, black-tipped ears and the pelage is in transition to the grey-white winter pelage. Right: The liver is paler than normal and displays a prominent lobular pattern. Photos: Aleksija Neimanis.

Microscopically, massive, acute hepatocellular necrosis and apoptosis were seen in the liver. Just as in EBHS (Gavier-Widén, 1994; Fuchs & Weissenbock, 1992), variable mineralization (likely mitochondrial calcification) and fatty degeneration of hepatocytes, inflammation (probable heterophils) and focal areas of lytic necrosis also were observed (Figure 11). It appears that mineralization may be a feature of lagoviral hepatitis in hares irrespective of the causative lagovirus, whereas it is not a typical feature of RHD in rabbits (Marcato *et al.*, 1991). Lagoviral antigen was seen within the cytoplasm and nucleus of hepatocytes and cytoplasm of Kupffer cells in the liver (Figure 11) and within the cytoplasm of macrophages in the spleen, again mirroring viral distribution in EBHS (Gavier-Widén, 1994).

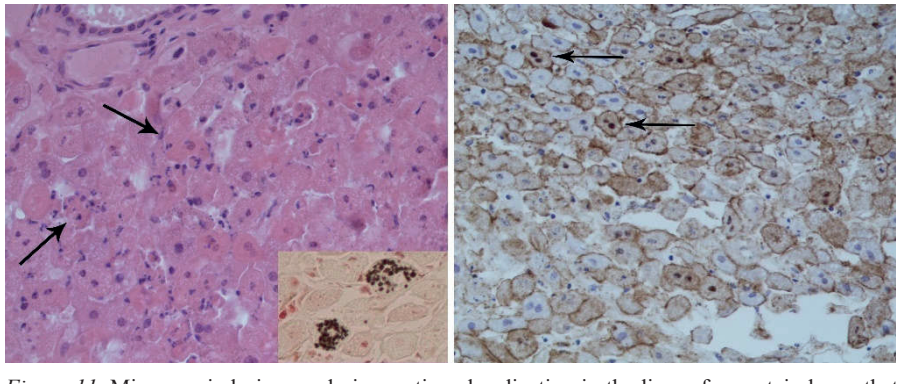

Figure 11. Microscopic lesions and virus antigen localization in the liver of mountain hares that died of GI.2/RHDV2 infection. Left: Hepatocellular necrosis and apoptosis. Arrows depict dead hepatocytes. HE 400X. In the insert, mineral deposits in hepatocytes are stained as black granules using von Kossa histochemical stain. Right: Immunohistochemical visualization of viral antigen

(positive areas stained brown) in hepatocytes. Arrows depict hepatocytes with prominent intranuclear staining. DAB 400X magnification. Photomicrographs: Aleksija Neimanis.

Genotyping and immunotyping of the lagovirus from all three hares confirmed infection with GI.2/RHDV2 rather than GII.1/EBHSV. No rabbits inhabit the island and the virus likely originated from a widespread outbreak of GI.2/RHDV2 on the adjacent mainland (Figure 8, section 4.1.2.). The VP60 gene sequences from mountain hares grouped together with a strain found in a rabbit that died of GI.2/RHDV2 in July 2016 in Falkenberg, approximately 50 km away (Figure 7, section 4.1.1.). How the virus arrived to the island is not known, but the island lies only 3 km from the adjacent mainland and hosts approximately 50 000 tourists during the summer months each year. Transfer via humans, insects or scavenging birds are all realistic possibilities. There was probably high environmental contamination of GI.2/RHDV2 on the mainland from the ongoing, largescale outbreaks in rabbits, and humans may have inadvertently brought contaminated material over, for example, on their shoes. In Australia, Wardang Island lies 5km off the mainland, which is further offshore than Hallands Väderö. Insects were believed to have spread GI.1/RHDV from Wardang Island to mainland Australia (Cooke & Fenner, 2002).

Notably, 4.5 months passed between the death of the first and last mountain hare received for examination and, if reports of dead mountain hares are also included, the mortality event lasted for up to six months in the absence of rabbits. This means that virus may have circulated amongst hares, may have persisted in the environment to re-infect new hares and/or may have been introduced more than once. Further investigation of GI.2/RHDV2 strains that were circulating in rabbits and hares using whole genome sequencing and subsequent Bayesian analysis coupled with continued monitoring of mountain hares can provide more insight. No evidence of GI.2/RHDV2 infection was found in six apparently healthy, hunted hares collected on the island during and after the mortality event. Further research is required to determine if mountain hares are competent maintenance hosts of GI.2/RHDV2, or if they are simply susceptible to spillover infections from rabbits.

The mountain hare is the fourth *Lepus* species documented to succumb to GI.2/RHDV2. Perhaps susceptibility in hares is common and development of disease is a question of exposure rather than susceptibility. Because GI.2/RHDV2 is an emerging virus, complete host range for this virus likely has not yet been defined. Minimizing exposure of new or vulnerable hare species and populations to GI.2/RHDV2 may be a warranted precautionary measure. Additionally, GI.2/RHDV2 should be considered when investigating unexplained mortality events in other lagomorph species that have had contact with wild or domestic European rabbits.

4.3 Pathology and virus tissue distribution of GI.2/RHDV2 in young and adult rabbits

Inoculated rabbits (study **III**) displayed clinical signs and gross and microscopic lesions that were consistent with RHD in adult rabbits infected with GI.1/RHDV viruses (Fuchs & Weissenbock, 1992; Marcato *et al.*, 1991). Fever was observed within 52 hpi and death (n = 4) or euthanasia because of terminal RHD (n = 3) occurred within 96 hpi, consistent with peracute or acute RHD (Marcato *et al.*, 1991; Xu & Chen, 1989). This differs from the longer disease course (mortality occurred 3-9 days post inoculation) and lower mortality rates (9% and 46% for strains 10-32 and 10-28, respectively) described for early strains of GI.2/RHDV2 (Le Gall-Recule *et al.*, 2013), and suggests that the BlMt-1 strain used in this study is more pathogenic. Similar findings were reported by Capucci *et al.* (2017) when studying more recent GI.2/RHDV2 strains in Italy. Capucci et al. (2017) suggest that just as for GI.1/RHDV (Elsworth *et al.*, 2014), selection pressure favours increased pathogenicity.

4.3.1 Five-week old kittens

In stark contrast to infection with GI.1/RHDV virus, kittens inoculated with GI.2/RHDV2 developed acute, fatal necrotizing hepatitis. Young rabbits, ranging from two to six weeks of age, experimentally infected with GI.1/RHDV show very mild, subclinical infection of few, scattered periportal hepatocytes accompanied by predominantly lymphocytic inflammation (Marques *et al.*, 2012; Prieto *et al.*, 2000; Mikami *et al.*, 1999). Both kittens examined microscopically in the current study had severe, massive lesions extending throughout the liver lobule with accompanying mild to moderate infiltrate of heterophils (Figure 12). Lymphocytic inflammation, proposed by Ferreira *et al.* (2005) to be an important factor in resistance to clinical disease, was conspicuously absent. Therefore, one of the key findings of the current study was that the Australian GI.2/RHDV2 strain invoked a different inflammatory response in young kittens than GI.1/RHDV viruses do. In this study, the inflammatory response in kittens was indistinguishable from that of adults infected with GI.1/RHDV or GI.2/RHDV2, supporting the hypothesis that a lymphocytic response plays an important role in outcome following infection.

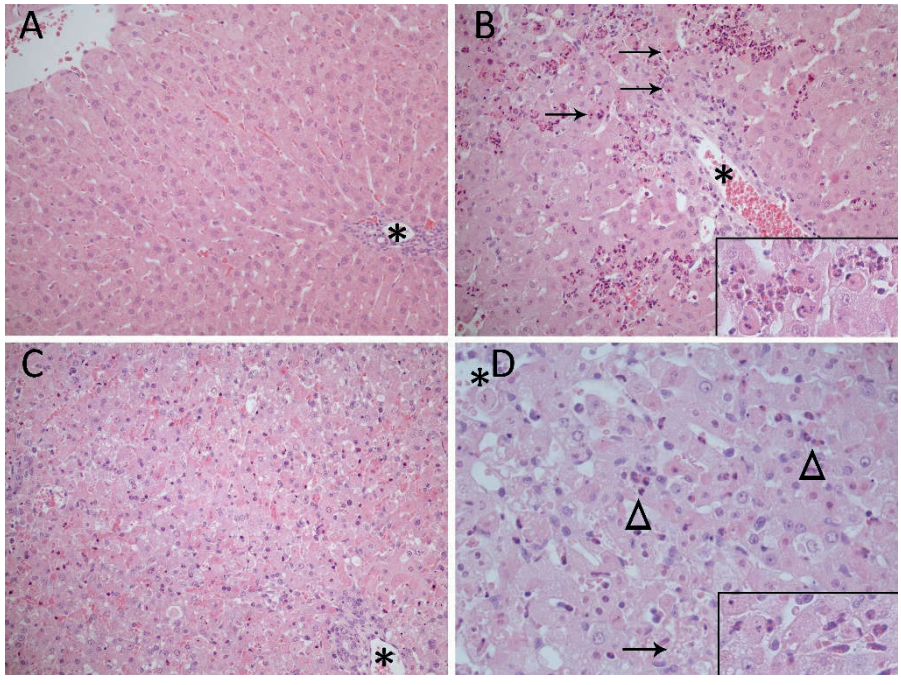

Figure 12. Microscopic lesions in the liver of rabbits (*Oryctolagus cuniculus*) inoculated with *Lagovirus europaeus* G1.2 (BlMt-1). Asterisks denote portal areas. Haematoxylin and eosin stain. A) Uninfected control rabbit (rabbit 130). 200X magnification. B) Earlier stage of acute infection in an adult rabbit (rabbit 283). There is degeneration and death of hepatocytes at the limiting plate and periportally surrounded by intensive heterophilic inflammation. Arrows denote apoptotic bodies. 200X magnification. Insert: high magnification of dead hepatocytes surrounded by heterophils. C) Terminal stage of acute infection in a 5 week old rabbit (rabbit 303). There is massive hepatocellular degeneration and death. 200X magnification. D) Terminal stage of acute infection in a 5 week old rabbit (rabbit 306). Arrowheads denote clusters of heterophils that are seen throughout the lobule and the arrow indicates an area of lytic necrosis. 400X magnification. Insert: high magnification of heterophils surrounding dead hepatocytes. Figure reproduced from Neimanis *et al.* (2018), Veterinary Research under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/).

Other microscopic lesions in these kittens that mimicked GI.1/RHDV infection in adults included multisystemic thrombi indicative of DIC, particularly within renal glomeruli, splenic necrosis, and lymphocytolysis in a variety of lymphoid tissues (Figure 13).

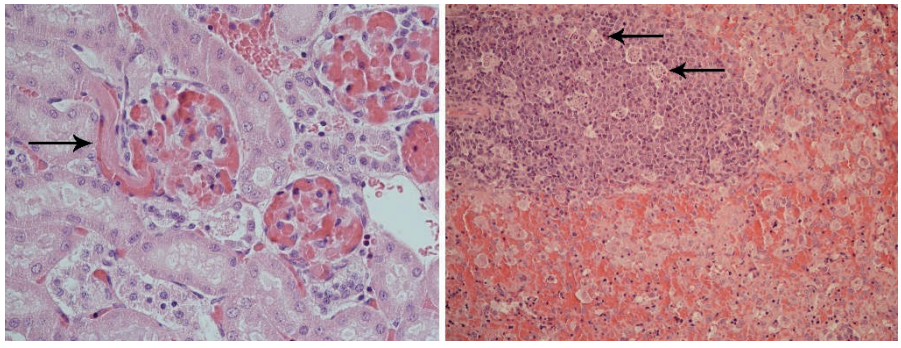

Figure 13. Micropscopic lesions in rabbit kittens inoculated with GI.2/RHDV2. Kidney (left): The glomeruli are congested and contain fibrin thrombi (arrow). Interstitial haemorrhage is seen at the top of the figure. HE 400X. Spleen (right): Severe lymphocytolysis is seen in the while pulp and arrows denote tingible body macrophages. Increased numbers of macrophages and necrosis are seen in the red pulp (bottom of figure). HE 200X. Photomicrographs: Aleksija Neimanis.

4.3.2 Adult rabbits

Based on virus tissue loads, time of euthanasia following inoculation (54 and 96 hpi) and comparison with GI.1/RHDV infections (Jung *et al.*, 2000; Park *et al.*, 1995), the two adults represented earlier and terminal stages of RHD, respectively. In the adult that was euthanized earlier, small to moderate numbers of dead hepatocytes were seen periportally and they were surrounded by marked heterophilic inflammation (Figure 12). In the adult with terminal RHD, severe hepatocellular apoptosis and necrosis were seen throughout the lobule. Although sample size was very small, results suggest that GI.2/RHDV2 progresses like GI.1/RHDV i.e. from few periportal hepatocytes towards the centre of the lobule. This zonal pattern of necrotizing hepatitis has also been observed in numerous field cases of GI.2/RHDV2 in Sweden (Figure 14).

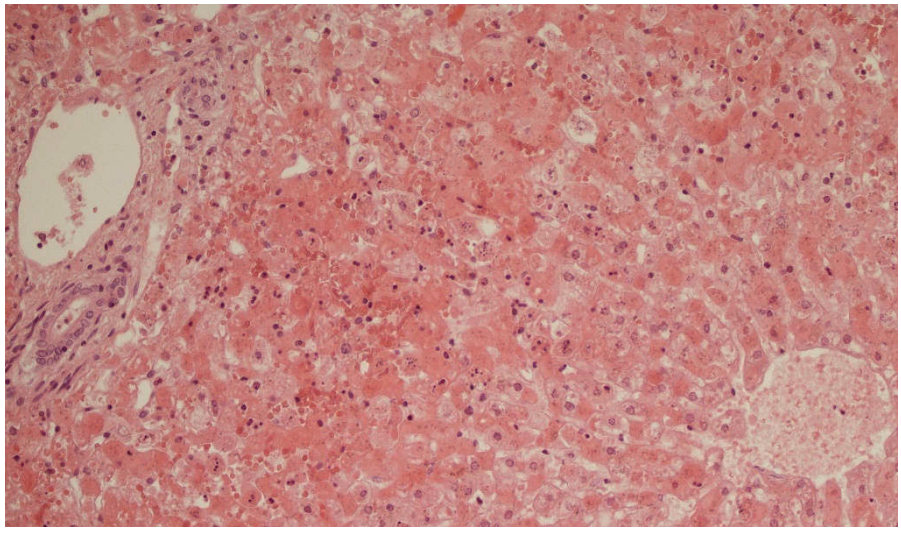

Figure 14. Periportal to midzonal necrosis in the liver of a rabbit that died from natural GI.2/RHDV2 infection. HE 200X. Photomicrograph: Aleksija Neimanis.

Another notable finding was seen in the bone marrow of the two adult rabbits. The M:E was lower in the rabbits euthanized at 54 hpi (0.99:1) and 96 hpi (0.47:1) compared to the control rabbit (2.08:1). This was coupled with a greater proportion of earlier versus later stage myeloid cells in inoculated rabbits versus the control. Earlier stage myeloid cells made up 16%, 24% and 55% of the myeloid cell population in the control rabbit, the rabbit euthanised at 54 hpi and the rabbit euthanised at 96 hpi, respectively. Thus, the adult rabbit that was euthanized 96 hpi had a very low M:E, and of the myeloid cells present, more than half were classified as earlier stage cells. This shift in relatively fewer myeloid precursors of which earlier stages predominated may reflect increased demand for mature myeloid precursors and heterophils. Additionally, signs of disrupted erythropoiesis (bi- and multinucleate rubricytes) were seen in the adult with terminal RHD and warrant further investigation.

4.3.3 Virus tissue distribution

Viral antigen was detectable by IHC in hepatocytes of all four inoculated rabbits examined microscopically. For the adult interpreted to be at an earlier stage of disease, liver was the only organ in which antigen was detected, supporting early targeting of the liver as the primary site of infection for GI.2/RHDV2. For the other three animals with terminal RHD, antigen was also observed in cells of the mononuclear phagocytic system, particularly in the liver (Kupffer cells) and spleen. The presence of antigen in the cytoplasm of macrophages in terminal

stages of the disease seen here may represent phagocytosis of disseminated viral material, but the role of macrophages in the pathogenesis of RHD requires further investigation. Notably, no viral antigen was seen associated with endothelium or lymphocytes in this material, suggesting that direct cytopathic effects by the virus on these cells are not responsible for DIC or lymphoid depletion, respectively.

Similar to findings for GI.1/RHDV (OIE, 2015), liver and serum contained the highest viral loads in terminal RHD (generally 10^8-10^9 viral copies per mg tissue) followed by the spleen (10^7-10^8 viral copies per mg tissue). Of note, serum contained three orders of magnitude less virus in the adult euthanised at an earlier stage of disease (10^5 viral copies per mg tissue), supporting the development of high viremia only after massive viral replication in the liver.

Quantification of viral tissue load (see Table 4 in paper **III**) also provided insight into pathogenesis and the sensitivity of the IHC. Intracellular virus was only detected in hepatocytes and cells of the mononuclear phagocytic system by IHC, suggesting presence of virus in other tissues reflects free virus in plasma or excreted into the lumen of organs such as intestine. However, high viral loads (at least 10^7 viral copies per mg tissue) were required before viral antigen could be detected by IHC, therefore antigen in cells and tissues containing lower levels of virus may not have been detected.

4.4 Challenges and limitations

While these studies provided new information on GI.2/RHDV2, data on disease impact were more challenging to obtain. Data collected from natural infections (studies **I**, **II** and **IV**) were dependent upon voluntary reporting and submission of dead animals. This underrepresentation of total cases, coupled with the lack of information on population sizes of wild leporids and number of domestic rabbits kept in Sweden, precluded quantification of impact of GI.2/RHDV2 in the host populations studied.

Sample sizes in studies **II**, **III** and **IV** were limited. As discussed above, studies **II** and **IV** relied on voluntary submissions, therefore sample size was limited by what was submitted. For the inoculation study (**III**), microscopic examination was only possible on four animals. While this puts limits on the definitive conclusions that can be drawn and precludes statistical analyses, the availability of high quality material coupled with previously published knowledge allowed for a detailed description of the histopathology, and for a number of hypotheses and conclusions to be generated. This would not have been possible using material from natural infections. Detailed microscopic evaluation of natural infections is often hampered and confounded by varying

degrees of post-mortem autolysis, concurrent pathology from other causes, and individual variation. This is especially problematic when working with free-ranging wildlife. In fact, autolysis was a limiting factor in the microscopic and IHC analyses performed in studies **II** and **IV**. Therefore, despite the small sample size in study **III**, new findings for GI.2/RHDV2 were generated that help validate observations reported from studies of GI.2/RHDV2 in the field and can help guide further investigations of natural disease.

In the inoculation study, bone marrow examination was conducted on paraffin-embedded, formalin-fixed tissue sections. While distinguishing cell morphology can be challenging on such material, examination was facilitated by the fact that rabbits have heterophils with easily discernible eosinophilic granules, rather than neutrophils present in other mammals. However, further investigations also incorporating concurrent cytological examination of bone marrow smears and complete blood counts are desirable. These additional analyses would also allow further exploration of the apparent dyserythropoiesis seen.

Evaluation of viral antigen distribution in tissues was challenging because of the lack of commercially available antibodies against lagoviruses. Donations of MAbs generated at the OIE Reference Laboratory for RHD for use in ELISAs made this work possible but required development and optimization of IHC methods. At the time of IHC development, GI.2/RHDV2 was still rapidly evolving and a set of MAbs that consistently recognised GI.2/RHDV2 was not available. A panlagovirus MAb cocktail was therefore used, necessitating typing of the virus by alternate methods. However, a more recent pool of MAbs specific for GI.2/RHDV2 has been donated and development of a GI.2/RHDV2 IHC is underway to serve as another diagnostic tool for the typing of pathogenic lagoviruses.

In addition, detection sensitivity of the IHC analysis in study **III** was low. Large amounts of virus needed to be present for detection of viral antigen *in situ*. This means that presence of lower levels of virus may have been missed. This is an important consideration if using this protocol for studies investigating, for example, entry routes of GI.2/RHDV2 in which viral loads may be considerably lower than in the main target organ of the liver. Additionally, to further knowledge on pathogenesis, methods to differentiate between phagocytised virus and replicating virus, such as *in situ* hybridization, are needed.

In study **III**, the strain chosen for the inoculation trial was the first GI.2/RHDV2 strain detected in Australia. It is a recombinant strain that is most closely related to a highly virulent strain circulating on the Iberian peninsula and was therefore considered relevant for both Australia and Europe. Because there appears to be wide variation in the pathogenicity and duration of disease of

different GI.2/RHDV2 strains, findings in study **III** may not apply to all GI.2/RHDV2 viruses. Further investigation into the pathology of other GI.2/RHDV2 strains therefore are warranted.

For the purposes of these studies, full sequences of the gene that codes for the VP60 capsid protein were used to infer phylogenetic relationships. While this is considered a robust gene for this purpose (Kinnear & Linde, 2010), recombination events in GI.2/RHDV2 have been recently described and are considered an important mechanism for generating genetic diversity (Lopes *et al.*, 2018; Lopes *et al.*, 2015). One major breakpoint is between the genes that encode for the non-structural and structural proteins. Although the VP60 capsid gene always comes from GI.2/RHDV2, the non-structural protein genes may originate from GI.1/RHDV or non-pathogenic lagoviruses. To better understand the phylogenetic relationships for GI.2/RHDV2 strains, analysis of the whole viral genome is desirable.

5 Conclusions and future perspectives

The emergence of GI.2/RHDV2 changed our understanding of pathogenic lagoviruses and necessitated re-examination of the rigid host species and age barriers documented for GI.1/RHDV and GII.1/EBHSV. Using a broad approach and combining tools from the fields of pathology, virology, molecular biology and epidemiology, results from this thesis help define and provide insight into this new, expanded paradigm of pathogenic lagoviruses. Two new host species, the European brown hare and the mountain hare, were added to the list of susceptible animals, and disease caused by GI.2/RHDV2 was indistinguishable from that caused by GII.1/EBHSV in these species. These studies on hares also provided insight into the disease ecology of GI.2/RHDV2 in different ecological contexts. Phylogenetic analysis supported spillover infection from rabbits, and in Spain and Italy, spillover events from rabbits to hares appeared to be infrequent and sporadic based on serological surveys. Further research in mountain hares is required to determine if they are competent hosts in the absence of rabbits. Investigation of pathology in young rabbits infected with GI.2/RHDV2 provided support for the protective role of a lymphocytic inflammatory response in young rabbits infected with GI.1/RHDV. Studies on pathology and viral tissue distribution of GI.2/RHDV2 in rabbits and hares suggest that the disease has the same pathogenesis as disease caused by GI.1/RHDV and GII.1/EBHSV in adult animals. The more recent GI.2/RHDV2 strain used in the inoculation study appears to be more pathogenic than early GI.2/RHDV2 strains, supporting the hypothesis that selection pressure on GI.2/RHDV2 favours increased pathogenicity. The first description of bone marrow lesions in RHD was provided and contributes to the understanding of the heteropenia described in GI.1/RHDV infections.

Results from this thesis also help track the global spread of GI.2/RHDV2 and provided information for domestic rabbit owners in Sweden so that they could apply measures to protect their animals from infection. Phylogenetic analyses of GI.2/RHDV2 circulating in Sweden suggests that the virus has evolved and/or

has been introduced more than once. Further research is required to measure impacts of GI.2/RHDV2 in rabbit and hare populations in Sweden. More rigorous and representative serosurveys on hunted animals can be used to estimate seroprevalence and can provide insight into how often sublethal exposure occurs. However, quantification of impact can only be done through intensive monitoring of leporid populations.

Findings in these studies also highlighted the need for continued surveillance and typing of circulating lagoviruses, as different viruses share similar clinical presentation and pathology. This information is needed to identify new viruses and variants that may impact wild populations or vaccination strategies of domestic animals. The list of new pathogenic and variably pathogenic lagoviruses continues to grow and we can expect continued emergence of new viruses. Applying knowledge gained from the re-emergence of RHD when GI.2/RHDV2 appeared can help better protect wild and domestic leporid populations to decrease the amount of losses seen for GI.2/RHDV2.

The work presented in this thesis is only the beginning of research on GI.2/RHDV2 performed in Sweden. This thesis began just before the widespread epizootics of GI.2/RHDV2 began in Sweden in 2016. Thanks to the on-going wildlife surveillance program and this project, we were able to follow GI.2/RHDV2 in Sweden from first introduction through the initial epizootics and collect a comprehensive set of tissues and data beyond the scope of this thesis. This will allow for further investigations on natural infections and virus evolution and change over time. For example, further examination of the genetic differences and any changes in pathology between pre-2016 strains and later strains may provide insight into the apparent differences in disease observed in the field. Additionally, as more GI.2/RHDV2 sequence data becomes publicly available and whole genome sequencing is more accessible, GI.2/RHDV2 origins and molecular epidemiology can be further explored.

Our understanding of pathogenic lagoviruses and lagoviruses in general is rapidly changing. Species and age barriers are less rigid for GI.2/RHDV2, and virulence has been shown to vary widely between strains and appears to change over time. Further investigation of different GI.2/RHDV2 strains, including recombinants, can help elucidate genotypic and phenotypic drivers of pathogenicity. In fact, G1.2/RHDV2 provides an excellent opportunity to study the factors responsible for the crossing of species, age and other host barriers and could provide important insight into how viruses acquire these traits and the mechanisms behind them.

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Popular science summary

Rabbit haemorrhagic disease (RHD) is a serious, highly contagious disease of wild and domestic rabbits. The disease was first detected in 1984 and has spread around the world. Rabbits die from liver disease and bleeding is often seen in internal organs. Death rate is usually high (90%) and rabbits die two to four days after infection. RHD is a viral infection caused by a lagovirus called *Lagovirus europaeus* GI.1/RHDV, or simply GI.1/RHDV. A closely related virus called GII.1/EBHSV causes a similar disease in hares known as European brown hare syndrome (EBHS). Young rabbits and hares do not get sick, and the rabbit virus only affects rabbits, while the hare virus only affects hares. Our understanding of these viruses changed when a new virus called GI.2/RHDV2 was found in rabbits in 2010. This rabbit virus also kills young rabbits and it affects two different kinds of hares in southern Europe, the Sardinian Cape hare and the Italian hare.

The first part of this thesis investigated the arrival and spread of this new virus in Sweden. The first known cases of GI.2/RHDV2 were in two wild rabbits that died on Gotland in May 2013. Only a handful of cases were detected in the following years. Then, in the spring of 2016, things suddenly changed. Large numbers of outbreaks were seen throughout the southern half of Sweden in both wild and domestic rabbits. Although many domestic rabbits were vaccinated against RHD, the vaccine available at the time did not fully protect rabbits from the new virus. GI.2/RHDV2 had been in Sweden for three years before causing large-scale outbreaks. Reasons for the sudden increase in cases in 2016 are not known, but one likely reason is that the virus had changed. It had evolved, or a new strain had been introduced into Sweden, or probably both.

The second part of this thesis investigated the disease caused by this new virus in young and adult rabbits and described the disease it caused in two other kinds of hares, the European brown hare and the mountain hare. Prior to these studies, it was not known that this new rabbit virus could infect and kill these species of hares. Disease caused by GI.2/RHDV2 looks the same in both young

and adult rabbits, and it looks just like EBHS in the European brown hare and mountain hare. Hares appear to get the infection from rabbits. The outbreak of GI.2/RHDV2 in mountain hares in Sweden lasted for months on an island without rabbits, but the virus most likely was brought over from infected rabbits on the mainland.

It is important to continue to monitor which kinds of lagoviruses we have in Sweden. This information can help to prevent or minimize disease in, for example, isolated wild hare populations, and to make sure that effective vaccines are available for domestic rabbits. This new rabbit virus can infect a larger number of species and more age groups than the classic rabbit and hare lagoviruses. Future research to uncover the reasons behind this could help us understand how viruses gain the ability to infect new hosts.

Populärvetenskaplig sammanfattning

Kaningulsot (rabbit haemorrhagic disease, RHD) är en mycket smittsam och allvarlig sjukdom hos vild- och tamkaniner. RHD upptäcktes först 1984 och har spridits runt om i världen. Drabbade kaniner dör av omfattande leverskador och man ser ofta blödningar i de inre organen. Dödligheten är vanligtvis hög (90%) och kaninerna dör inom två till fyra dagar efter att de infekterats. RHD är en virussjukdom som orsakas av ett lagovirus; *Lagovirus europaeus* GI.1/RHDV, eller förkortat; GI.1/RHDV. Ett närbesläktat virus som kallas för GII.1/EBHSV orsakar fältharesjuka (även kallad European brown hare syndrome, EBHS), som är en liknande sjukdom hos harar. Unga kaniner och harar blir inte sjuka, och kaninviruset påverkar bara kaniner, medan harviruset endast påverkar harar. Vår uppfattning om dessa virus ändrades när ett nytt virus som heter GI.2 / RHDV2 upptäcktes hos kaniner 2010. Till skillnad från GI.1/RHDV så dödar GI.2/RHDV2 även unga kaniner och drabbar också två olika sorters harar i södra Europa (kaphare och italiensk hare).

Avhandlingens första del undersöker introduktionen och spridningen av det nya kaningulsotsviruset i Sverige. De första kända fallen av GI.2/RHDV2 upptäcktes hos två vilda kaniner som dog på Gotland i maj 2013. Endast enstaka fall av det här nya viruset upptäcktes i Sverige de följande åren. Sjukdomsläget ändrades plötsligt våren 2016. Ett stort antal utbrott rapporterades i de södra delarna av Sverige hos både vild- och tamkaniner. Även om många tamkaniner vaccinerades mot RHD, så gav de tillgängliga vaccinerna i bästa fall enbart delvis skydd mot det nya viruset. GI.2/RHDV2 hade varit konstaterad i Sverige i tre år innan det orsakade stora utbrott. Orsaken till ökningen av antal fall 2016 är okänd, men en trolig förklaring är att viruset hade förändrats. Viruset hade antingen utvecklats, eller så kom en ny stam av GI.2/RHDV2 till Sverige, eller så skedde både och.

Avhandlingens andra del undersöker sjukdomen som orsakas av det nya kaningulsotsviruset hos unga och vuxna kaniner och hos ytligare två sorters harar, fälthare och skogshare. Dessa harar var tidigare inte kända värddjur för

det nya viruset. Sjukdomen som orsakas av GI.2/RHDV2 ser likadan ut hos både unga och vuxna kaniner, och liknar fältharesjuka hos fältharar och skogsharar. Harar verkar smittas av kaniner. Utbrottet av GI.2/RHDV2 hos skogsharar i Sverige pågick under flera månader på en ö utan kaniner, men viruset har sannolikt överförts från smittade kaniner på fastlandet.

Det är viktigt med fortsätt övervakning av vilka typer av kaningulsotsvirus vi har i Sverige. Informationen kan bidra till att förebygga eller minimera sjukdomen i exempelvis isolerade populationer av harar samt för att säkerställa att effektiva vacciner finns tillgängliga för tamkaniner. Detta nya kaningulsotsvirus kan smitta ett större antal arter och fler åldersgrupper än klassiska lagovirus. Framtida forskning som är inriktad på virusets förmåga att smitta nya arter och åldersgrupper kan hjälpa oss att förstå hur virus smittar nya värdar.

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Appendix 1.

Current and proposed new names for lagoviruses according to Le Pendu *et al*. (2017) referred to in this thesis.

Current name	Proposed, new name
Rabbit haemorrhagic disease virus (RHDV)	*Lagovirus europaeus* GI.1
RHDVa	*Lagovirus europaeus* GI.1a
RHDV2 or RHDVb	*Lagovirus europaeus* GI.2
Rabbit calicivirus (RCV)	Unclassified*
RCV-E1	*Lagovirus europaeus* GI.3
RCV-A1	*Lagovirus europaeus* GI.4
Michigan RCV	Unclassified*
European brown hare syndrome virus (EBHSV)	*Lagovirus europaeus* GII.1
RHDV G1 (paper III)	*Lagovirus europaeus* GI.1b
Recombinant of RHDV G1 and RHDV2, recombinant breakpoint between the polymerase and capsid coding regions (paper III)	*Lagovirus europaeus* GI.1bP-GI.2

*Unclassified until sufficient strains are available for phylogenetically supported assignment to a subgroup